Investigating the antimicrobial effect of Rosmarinus officinalis leaf extract on enteropathogens isolated from raw eggs

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

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CERTIFICATE

TO WHOM IT MAY CONCERN

This is to certify that Ms. Sayanti Mazumder, student of M.Sc. (Biotechnology) - Academic session 2023-25, of Sister Nivedita University, Kolkata, conducted her dissertation project entitled "Investigating the antimicrobial effect of Rosmarinus officinalis leaf extract on enteropathogens isolated from raw eggs" under my supervision. Her effort was extremely appreciable and satisfactory. We wish Sayanti best for all of her future endeavours.

Yours truly,

Sanmitra Ghosh.

28/05/2025

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This is to certify that the project report titled " Investigating the antimicrobial effect of Rosmarinus officinalis leaf extract on enteropathogens isolated from raw eggs ", submitted by Ms. Sayanti Mazumder (Registration No. 230020107872, Enrollment No.2322210002010) to the Department of Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by her for the partial fulfilment of the requirements for the Master of Science degree.

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "Investigating the antimicrobial effect of Rosmarinus officinalis leaf extract on enteropathogens isolated from raw eggs" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology is an authentic record of my own work carried out during the period from 13th January 2025 to 8th April 2025 under the supervision of Dr. Sanmitra Ghosh, assistant professor of Department of Biological Sciences, Adamas University.

I acknowledge that I have read and understood the UGC regulations, 2018 (*Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institutions*).

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Sayanti Mazumder

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ABBREVIATIONS

AMR	Antimicrobial Resistance
HEA	Hektoen Enteric Agar
MHA	Mueller Hinton Agar
TSI	Triple Sugar Iron
EMB	Eosin Methylene Blue
TPC	Total Polyphenol Content
RSA	Radical Scavenging activity
DPPH	2,2-diphenyl-1-picrylhydrazyl
CV	Crystal Violet
SPR	Surface Plasmon Resonance
OD	Optical Density
AgNPs	Silver Nanoparticles
BST	Barasat
BKP	Barrackpore
NAL	Nalikul
BD	Bardhaman

ABSTRACT

This study explores the extent of microbial contamination present in raw eggs sourced from various local markets across West Bengal, with an emphasis on detecting enteric pathogens such as *Escherichia coli* and *Salmonella* species. Isolation and identification of bacterial contaminants were performed using standard microbiological culturing techniques, followed by Gram staining and a series of biochemical characterization tests. The findings revealed a high prevalence of *E. coli* among the samples, alongside the detection of one confirmed isolate of *Salmonella*. Further analysis through antibiotic susceptibility testing demonstrated significant resistance patterns, particularly against several commonly prescribed antibiotics. These results highlight the growing concern of antimicrobial resistance (AMR) within the food supply chain, posing substantial public health risks.

In an effort to propose alternative antimicrobial strategies, the study evaluated the antibacterial potential of *Rosmarinus officinalis* (rosemary) leaf extract and its green-synthesized silver nanoparticle (AgNP) formulation. Preliminary phytochemical screening confirmed the presence of various bioactive constituents in the crude extract, known for their therapeutic and antimicrobial properties. Assessment of antimicrobial activity revealed that while the crude rosemary extract exhibited moderate inhibitory effects against the isolated pathogens, the nanoparticle formulation demonstrated significantly enhanced efficacy. Particularly, the rosemary-based AgNPs showed superior capability in disrupting bacterial biofilms, which are critical in bacterial persistence and resistance mechanisms.

These findings suggest that *Rosmarinus officinalis*-derived silver nanoparticles possess promising antimicrobial properties that could serve as a natural and effective alternative to conventional synthetic antimicrobial agents. Incorporating such green nanotechnological approaches could play an important role in improving food safety and managing infections caused by multidrug-resistant pathogens. This study underscores the urgent need for the development of novel, plant-based antimicrobial interventions to combat the rising threat of AMR, particularly in foodborne bacterial contamination.

KEYWORDS

Antibiotic-resistant bacteria, biofilm control, botanical antimicrobial agents, contaminated eggs, herbal-based nanoparticles, intestinal pathogens, plant-derived phytochemicals.

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LITERATURE REVIEWS

1. **Davies, J., & Davies, D. (2010)**
   Microbiology and Molecular Biology Reviews, 74(3), 417–433.

   This foundational review by Davies and Davies highlights the global issue of antimicrobial resistance (AMR) and its mechanisms, such as the evolution of resistance genes in bacteria. The authors explain how antibiotic overuse and misuse have led to resistant strains, which is a central issue in your research. This paper underscores the need for alternative strategies, including plant-based antimicrobials and nanotechnology, to combat drug-resistant pathogens. This is particularly relevant to your study, which explores the use of silver nanoparticles (AgNPs) and rosemary as potential solutions to AMR in foodborne pathogens like Salmonella.

2. **Gantois, I. et al. (2009)**
   FEMS Microbiology Reviews, 33(4), 718–738.

   Gantois et al. explore the mechanisms by which Salmonella Enteritidis contaminates eggs, focusing on how the bacteria can survive inside the egg, either through vertical transmission from hen to egg or through contamination via the egg shell. This study is vital to your research, as it explains the potential pathways of contamination that could lead to Salmonella presence in raw eggs, which is the focus of your pathogen isolation study. Understanding these contamination routes helps contextualize the importance of identifying and controlling Salmonella in eggs, which is a significant part of your dissertation.

3. **Li, R. et al. (2023)**
   Frontiers in Microbiology, 14, 1283835.

   Li et al. (2023) provide a recent, in-depth genomic analysis of Salmonella strains isolated from eggs in China. This study is particularly relevant because it uses genomic techniques to profile Salmonella, identifying virulence factors and antimicrobial resistance genes. Their findings show resistance mechanisms like extended-spectrum beta-lactamases (ESBLs), which are crucial for understanding the resistance patterns of Salmonella strains in your study. This paper strengthens the background of your research by highlighting the genetic factors involved in Salmonella resistance and helps contextualize the need for alternative antimicrobial approaches, such as the use of AgNPs and herbal extracts.

4. **Rai, M. et al. (2012)**
   Journal of Applied Microbiology, 112(5), 841–852.

   Rai et al. examine the antimicrobial properties of silver nanoparticles (AgNPs), particularly against multidrug-resistant bacteria. This study is directly relevant to your research, as it provides scientific backing for the efficacy of AgNPs in combating resistant strains. The authors explain how AgNPs disrupt bacterial cell membranes, generate reactive oxygen species (ROS), and interfere with bacterial DNA replication, leading to cell death. These mechanisms are key to your study's hypothesis that AgNPs can be effective against Salmonella and other enteric pathogens, making this paper an essential part of your literature review.

5. **Sharma, D. et al. (2020)**
   Materials Letters, 263, 127261.

   Sharma et al. describe an environmentally friendly (green) method for synthesizing silver nanoparticles using plant extracts, an approach you adopt in your research with rosemary. Their study demonstrates that plant-based methods not only produce stable nanoparticles but also offer a sustainable and low-toxicity alternative to chemical synthesis. This research supports your methodology of using rosemary extract as a source for AgNPs synthesis, highlighting both the ecological benefits and the effectiveness of green synthesis methods in antimicrobial applications.

6. **Singh, R., & Shushni, M. A. M. (2014)**
   Asian Pacific Journal of Tropical Biomedicine, 4(6), 456–460.

   Singh and Shushni's work on the phytochemical profile and antimicrobial activity of Rosmarinus officinalis (rosemary) is directly relevant to your study. They identify several bioactive compounds in rosemary, including rosmarinic acid, carnosic acid, and essential oils, which are responsible for its antibacterial properties. Their study confirms the effectiveness of rosemary as a natural antimicrobial agent, validating its selection in your research. This work supports your hypothesis that rosemary can be used not only for its direct antimicrobial properties but also as a source for synthesizing AgNPs with enhanced antibacterial activity.

7. **Kora, A. J., & Rastogi, L. (2013)**
   International Journal of Nanomedicine, 8, 409–419.

   Kora and Rastogi explore the potential of plant-derived silver nanoparticles in antimicrobial applications, demonstrating their efficacy against a variety of pathogens. They focus on the antimicrobial action of nanoparticles synthesized using plant extracts, similar to the methodology in your dissertation. The study reinforces your choice to use rosemary for AgNP synthesis, as it highlights the effectiveness of plant-based nanoparticles against both Gram-positive and Gram-negative bacteria. This paper supports your strategy of combining rosemary's antimicrobial properties with AgNPs to enhance pathogen control in raw egg extracts.

8. **Singh, S. et al. (2019)**
   Food Control, 100, 219–224.

   Singh et al. investigate the prevalence and antibiotic resistance of Salmonella strains isolated from poultry eggs in India. This study provides essential data on the occurrence of Salmonella in eggs and the resistance patterns observed in these strains, which are critical to your work on pathogen isolation from raw egg extracts. The findings support the need for alternative antimicrobial approaches, as they highlight the widespread presence of multidrug-resistant Salmonella. This research helps to contextualize your study in the Indian context, where Salmonella prevalence and resistance are significant concerns.

9. **El-Beltagy, A. E., & El-Far, A. H. (2018)**
   Veterinary World, 11(9), 1292–1297.

   El-Beltagy and El-Far's review of the biological and antimicrobial properties of rosemary strengthens the rationale for using rosemary in your study. They provide evidence of rosemary's broad-spectrum antibacterial effects, particularly against Gram-negative and Gram-positive bacteria. Their findings support your decision to incorporate rosemary not only as a plant-based antimicrobial but also as a source for AgNP synthesis. Rosemary's ability to inhibit bacterial growth and biofilm formation aligns with your objectives of evaluating its effectiveness in controlling Salmonella and other enteric pathogens.

10. **Shafreen, R. M. et al. (2021)**
    Environmental Research, 197, 111210.

    Shafreen et al. provide a comprehensive review of plant-derived nanoparticles, focusing on their potential to address AMR. This paper discusses various plant extracts used for nanoparticle synthesis and their antimicrobial properties. The authors highlight the advantages of using plant-based nanomaterials, such as reduced toxicity and enhanced activity against resistant pathogens. This work supports your use of rosemary-mediated silver nanoparticles in your study, confirming the growing interest in green nanotechnology and its role in combating AMR. The review further validates the scientific basis for your research, which aims to explore the dual antimicrobial action of rosemary and AgNPs.

11. **Zong, Z. et al. (2018)**
    Antimicrobial Agents and Chemotherapy, 62(4), e02265-17

    Zong et al. investigate the use of silver nanoparticles (AgNPs) in combination with conventional antibiotics to overcome multidrug-resistant (MDR) bacteria. This study focuses on the synergistic effects of AgNPs and antibiotics against resistant pathogens, providing evidence that AgNPs enhance the antibacterial activity of traditional antibiotics. This paper is significant for your research because it supports the idea that silver nanoparticles could be a powerful tool in tackling antibiotic-resistant Salmonella, especially in combination with herbal extracts like rosemary. The synergistic effect can be a promising strategy for addressing AMR, which is a major focus of your study.

12. **Parvez, S. et al. (2019)**
    Antioxidants, 8(8), 285

    Parvez et al. explore the antioxidant and antimicrobial properties of rosemary, particularly focusing on its polyphenolic compounds like rosmarinic acid. Their research indicates that rosemary extract has potent antimicrobial and antioxidant activities, making it a strong candidate for controlling microbial growth in foodborne pathogens. This is highly relevant to your study, where rosemary’s dual antimicrobial and antioxidant properties can play a role in not only inhibiting Salmonella growth but also enhancing the stability of silver nanoparticles. The paper supports the rationale for using rosemary extract in your nanoparticle synthesis and antimicrobial testing.

1. INTRODUCTION:

Enteric bacteria comprise a broad category of microorganisms that primarily reside in the gastrointestinal tracts of humans and animals. While many of these microbes contribute positively to gut health as part of the normal intestinal flora, some species are pathogenic and pose significant health risks. Pathogenic enteric bacteria, such as *Escherichia coli*, *Salmonella* spp., and *Shigella* spp., are known to cause gastrointestinal diseases. These infections can range in severity from mild digestive discomfort to severe systemic illness, particularly in at-risk groups such as young children, the elderly, and immunocompromised individuals.

These microorganisms are not limited to the gastrointestinal tract; they are commonly found in various environments, including water bodies, soil, plants, animal hosts, and food sources. Foodborne transmission remains one of the most prevalent pathways through which these pathogens enter the human body. Eggs, in particular, are considered a high-risk food item for enteric bacterial contamination. Contamination can occur in two main ways: internally, when bacteria infect the reproductive organs of the hen and are deposited inside the egg during formation, and externally, when fecal matter or other contaminants come into contact with the eggshell after laying. The porous nature of eggshells allows bacteria to penetrate and reach the interior content, potentially.

Both commercially farmed and free-range eggs are vulnerable to such contamination. Factors such as unhygienic handling, poor sanitation during collection, and inadequate storage conditions can further increase the microbial load. With the global consumption of eggs steadily rising, especially in areas with limited regulatory control, safeguarding the microbiological quality of eggs has become a crucial public health concern.

Compounding this issue is the increasing resistance of enteric pathogens to commonly used antibiotics. The widespread and often inappropriate use of antibiotics in agriculture and clinical settings has led to the evolution of multidrug-resistant strains. As a result, traditional antimicrobial therapies are becoming less effective, necessitating the development of alternative approaches to infection control.

One promising avenue of research involves the use of herbal extracts with antimicrobial properties. Traditional medicine systems have long utilized plant-based remedies for treating infections, and modern studies have begun to validate these practices. Many plant-derived compounds have demonstrated effectiveness against a range of bacterial pathogens, including those responsible for enteric infections. Notable examples include extracts from neem (*Azadirachta indica*), garlic (*Allium sativum*), turmeric (*Curcuma longa*), rosemary (*Rosmarinus officinalis)* and tulsi (*Ocimum sanctum*), which have shown inhibitory effects on *Salmonella*, *E. coli*, and *Shigella* species. These natural agents act through multiple mechanisms such as disrupting bacterial cell membranes, inhibiting protein synthesis, and interfering with microbial communication systems like quorum sensing.

Due to their natural origin, lower potential for toxicity, and reduced likelihood of fostering antibiotic resistance, herbal antimicrobials offer a sustainable and environmentally friendly alternative to conventional antibiotics. Integrating such botanical solutions into food safety practices, particularly in the context of egg hygiene, could play a pivotal role in controlling the spread of enteric infections while simultaneously addressing the challenge of antibiotic resistance.

2. MATERIALS AND METHODS:

2.1. Collection of Egg Samples:

To investigate the presence of enteric pathogens in eggs obtained from various market environments, ten raw egg samples were collected from different regions across West Bengal. The sampling strategy encompassed diverse geographic locations—including urban, semi-urban, and rural areas—to capture a wide range of potential microbial diversity. Specifically, the sampling included **one egg from Barasat, two eggs from Nalikul, three eggs from Bardhaman, and four eggs from Barrackpore**. All samples were collected and transported under aseptic conditions to prevent external contamination during handling and processing.

2.2. Sample Preparation and Microbiological Plating:

All preparation and microbiological procedures were conducted in sterile conditions within a laminar airflow cabinet to maintain sample integrity. The following steps were followed:

1. **Media Preparation**: Hektoen Enteric (HE) agar (50 mL) and peptone buffer (20 mL) were freshly prepared for microbial assessment.
2. **Egg Content Collection**: Each egg was aseptically cracked, and the internal contents—excluding the yolk—were transferred into a sterile, transparent bag.
3. **Eggshell Processing**: The eggshells were crushed using a sterile mortar and pestle and placed in separate sterile bags.
4. **Dilution with Buffer**: To each sample bag (both internal content and shell), 10 mL of sterile peptone buffer was added.
5. **Homogenization and Filtration**: The mixtures were homogenized thoroughly and left to settle. The suspensions were then filtered through sterile filter paper into clean, sterile test tubes.
6. **Enrichment:** The filtered samples were incubated at 37°C for 24 hours to promote bacterial growth.
7. **Plating on Selective Media:** A 0.1 mL aliquot from each enriched filtrate was inoculated onto HE agar plates using the spread plate technique.
8. **Incubation and Colony Observation**: The inoculated plates were incubated at 37°C for 24–48 hours. Plates were then observed for the development of typical enteric bacterial colonies.
9. **Sub-culturing for Purity**: Distinct colonies showing typical morphological features on HE agar were picked using a sterile loop and streaked onto Mueller Hinton Agar (MHA) plates to obtain pure bacterial isolates.
10. **Final Incubation**: The MHA plates were incubated at 37°C for another 24 hours to ensure isolated growth for further microbiological analyses.

2.3 Gram Staining:

Gram staining was employed to categorize bacterial isolates based on differences in their cell wall structures. The procedure was performed as follows:

1. Distinct colonies were selected from incubated agar plates.
2. A thin smear of each bacterial isolate was prepared on a clean glass slide and allowed to air-dry.
3. The smear was heat-fixed by gently passing the slide through a flame.

4. Crystal violet stain was applied for 1 minute, followed by rinsing with distilled water.
5. Gram's iodine was then added to the smear and allowed to act for 1 minute before rinsing.
6. Decolorization was performed using ethanol or acetone-alcohol for 10–15 seconds, immediately followed by rinsing with distilled water.
7. Safranin was used as a counterstain and was applied for 1 minute before a final rinse.
8. The stained slides were left to air-dry and then examined under a microscope using oil immersion at 40x magnification.

2.4 Biochemical Characterization of Bacterial Isolates:

2.4.1. Triple Sugar Iron (TSI) Agar Test:

The TSI agar test was conducted to differentiate bacteria based on their ability to ferment sugars and produce hydrogen sulfide:

1. TSI agar was prepared and sterilized by autoclaving at 121°C for 15 minutes.
2. Under aseptic conditions, the sterile medium was dispensed into Petri dishes and allowed to solidify.
3. Two distinct colonies exhibiting different pigmentation (blue-green and orange) from HE agar were inoculated onto TSI agar using a sterile inoculating loop.
4. The inoculated plates were incubated at 37°C for 24 hours.
5. After incubation, pH variations in the medium were recorded using sterile pH indicator strips to determine acid or alkaline reactions.

2.4.2. Eosin Methylene Blue (EMB) Agar Test:

This test was used to identify lactose fermenters based on characteristic colony appearance:

1. EMB agar was prepared, sterilized, and poured into sterile Petri dishes and slant tubes.
2. Orange-pigmented colonies from HE agar were streaked onto the EMB medium.
3. The media were incubated at 37°C for 24 hours.
4. Plates and slants were examined for colony characteristics suggestive of lactose fermentation, such as dark-centered or metallic sheen colonies.

2.4.3. Urease Test:

The urease test was performed to detect the ability of bacteria to hydrolyze urea:

1. Urea agar slants containing urea and phenol red as a pH indicator were prepared and sterilized.
2. The test organisms were streaked in a zig-zag manner across the surface of the slants using a sterile loop.
3. The inoculated slants were incubated at 37°C for 24 hours.
4. Development of a pink color indicated a positive urease reaction, resulting from an alkaline shift in pH.

2.4.4. IMViC Tests:

The IMViC series—Indole, Methyl Red, Voges-Proskauer, and Citrate tests—were carried out for further biochemical profiling:

2.4.4.1. Indole Test-

Tryptone broth was inoculated with the test isolate and incubated at 37°C for 24–48 hours. Following incubation, 3–5 drops of Kovac's reagent were added. The formation of a red ring at the surface indicated a positive result.

2.4.4.2.Methyl Red (MR) Test-

MR-VP broth was inoculated and incubated at 37°C for 48 hours. Afterward, 5 drops of methyl red indicator were added. A red coloration signified strong acid production and a positive result.

2.4.4.3.Voges-Proskauer (VP) Test-

The same broth used for the MR test was treated with 15 drops of Barritt's A reagent (α-naphthol) and 5 drops of Barritt's B reagent (potassium hydroxide). The tube was gently shaken and allowed to stand for 15–30 minutes. A red color indicated a positive VP reaction.

2.4.4.4.Citrate Utilization Test-

Simmons citrate agar slants were lightly inoculated and incubated at 37°C for 24–48 hours. A change in color from green to blue, along with visible growth, was indicative of citrate utilization.

2.5 Antibiotic Susceptibility Testing via Disc Diffusion:

The antimicrobial resistance profile of the bacterial isolates was evaluated using the standard disc diffusion method:

1. Mueller Hinton agar was sterilized by autoclaving at 121°C and 15 psi for 15 minutes.
2. The medium was poured into four pre-labelled sterile Petri dishes and left to solidify for 15–20 minutes.
3. A sterile cotton swab was used to pick a single colony from a cultured isolate and spread evenly across the agar surface.
4. After letting the surface dry (5–10 minutes), antibiotic discs were aseptically placed on the agar. Discs included Kanamycin, Penicillin G, Ampicillin, Chloramphenicol, Streptomycin, Vancomycin, Nalidixic Acid, Gentamycin with a sterile blank disc serving as a negative control.
5. The plates were incubated at 37°C overnight, and the diameter of inhibition zones was measured to determine bacterial susceptibility.

2.6 Collection and Preparation of Plant Extract:

1. Fresh rosemary leaves were sourced from a super market and washed under running tap water.
2. The leaves were dried in a hot air oven for 5–6 hours.
3. Dried leaves were ground into a fine powder using an electric blender and stored in 50 mL Falcon tubes.
4. For extraction, 2 g of the powdered leaves was soaked in 20 mL of absolute ethanol in 15 mL sterile Falcon tubes and left undisturbed for 24 hours.

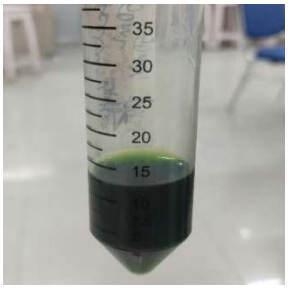


Fig 1: Rosemary leaves ethanolic extraction

5. The mixture was filtered the next day using Whatman filter paper, and the filtrate was collected for further use.

2.7 Antimicrobial Activity of Plant Extract:

The antimicrobial effect of rosemary extract was assessed against *E. coli* and *Salmonella spp.* using the agar well diffusion method:

1. Serial dilutions of the extract (1/2, 1/4, 1/6, 1/8) were prepared.
2. Sterilized MHA (15 mL) was poured into Petri plates and allowed to solidify, followed by appropriate labelling.
3. 70 µL of each overnight culture was spread on agar surfaces using a sterile glass spreader to achieve uniform growth, then left to dry for 10 minutes.
4. Wells were created using a sterile borer. Each well was filled with different dilutions of the extract, the undiluted extract (mother stock), and ethanol as the control.
5. Plates were left at room temperature for 30 minutes to allow diffusion, followed by incubation at 37°C for 24 hours.
6. Zones of inhibition were measured to evaluate antibacterial efficacy**.**

2.8 Phytochemical Screening of Rosemary Leaf Extract:

2.8.1. Qualitative Analysis:

2.8.1.1. Alkaloids:

1 mL of methanolic extract was mixed with 25% ammonia, followed by chloroform. After mixing, Mayer's reagent was added to observe precipitate formation, indicating alkaloids.

2.8.1.2. Flavonoids (Alkaline Reagent Test):

2 mL of methanolic extract was treated with a few drops of 1N NaOH (deep yellow color indicates flavonoids). Acidification with dilute HCl resulting in a color change confirms presence.

2.8.1.3. Terpenoids:

1 mL extract + 2 mL chloroform + 3 mL concentrated H_2SO_4. A reddish-brown layer at the interface indicates terpenoids.

2.8.1.4. Tannins:

1 mL extract + a few drops of 1% $FeCl_3$. A blackish-green color indicates tannins.

2.8.1.5. Saponins:

1 mL extract + 5 mL hot water, shaken vigorously. Stable foam formation after adding dilute HCl indicates saponins.

2.8.2. Quantitative Estimations:

2.8.2.1. Total Phenolic Content (TPC):

Measured using Folin-Ciocalteu reagent and expressed as mg GAE/g DW based on a gallic acid standard curve.

- 1 mL extract + 125 µL Folin reagent + 1975 µL water → after 1 min → add 375 µL of 20% Na_2CO_3 → final volume adjusted with water to 2500 µL.

- Incubated for 30 minutes; absorbance recorded at 760 nm.

2.8.2.2. Radical Scavenging Activity (DPPH Assay):

The antioxidant capacity was evaluated using the DPPH method.

- 0.004% DPPH in methanol was prepared.
- Varying extract concentrations (10–50 mg/mL) were mixed with methanol to 3 mL total volume, followed by 1 mL DPPH.
- Control: 3 mL methanol + 1 mL DPPH. Blank: 4 mL methanol.
- After 30 minutes of incubation, absorbance was measured at 517 nm.
- %RSA = [(A_control − A_sample)/A_control] × 100

2.9 Green Synthesis of Silver Nanoparticles (AgNPs):

1. 25 g of dried rosemary powder was added to 200 mL distilled water and boiled until reduced to ~50 mL.
2. After cooling, the extract was filtered using Whatman No. 1 filter paper.
3. Equal volumes of extract and 1 mM silver nitrate solution were mixed (1:1).
4. The mixture was incubated in a shaker for 4 hours at room temperature.
5. A visible color change confirmed nanoparticle synthesis.
6. The solution was centrifuged at 10,000 rpm for 15 minutes.
7. The pellet was resuspended in 10 mL sterile distilled water and stored at 4°C.

2.10 Antimicrobial Activity of Plant Extract and Nanoparticles:

1. Rosemary extract and AgNPs were tested at 1/2 and 1/8 dilutions.
2. Mueller Hinton agar (50 mL) was poured into sterile Petri plates and solidified, then labelled.
3. 75 µL of *E. coli* and *Salmonella spp.* cultures were spread evenly on the plates.
4. Wells were made, and respective dilutions of extract, AgNPs, and ethanol (control) were added.
5. After 30 minutes of diffusion at room temperature, plates were incubated at 37°C for 24 hours.
6. Zones of inhibition were recorded.

2.11 Preformed Biofilm Disruption Assay:

Day 1:

1. Nutrient broth with 1% glucose was prepared and autoclaved.
2. Crystal violet (0.1%) and 10X PBS were prepared.
3. Sterile Petri plates were filled with broth and inoculated with 100 µL of overnight bacterial cultures.
4. Plates were incubated for 48 hours:
    - Control (only culture)
    - Culture + extract
    - Culture + nanoparticle

Day 3:

1. After incubation, media was removed and plates were gently washed with 1 mL PBS.
2. Plates were air-dried, stained with 0.1% CV for 10 minutes, and dried again.
3. CV was solubilized with 1.5 mL 30% glacial acetic acid.
4. 1 mL of this solution was transferred to labeled Eppendorf tubes.
5. Absorbance was measured at 600 nm using UV-vis spectrophotometry.

2.12 Biofilm Inhibition Assay:

Day 1:

1. Nutrient broth with 1% glucose was prepared.
2. Plates were prepared as before and inoculated with 100 µL of bacterial culture.
3. A control plate without culture was included.
4. Treatment groups:
    - Culture only
    - Culture + extract
    - Culture + nanoparticle
5. Incubation was conducted at 37°C, and observations were recorded at 24, 48, 72, and 96 hours.

Day 2–5:

1. At each time point, biofilms were washed with PBS, stained with CV, dried, and solubilized in 1.5 mL glacial acetic acid.
2. 1 mL of each sample was transferred to Eppendorf tubes.
3. Absorbance was recorded at 600 nm.
4. Uninoculated samples served as blanks.

3. RESULTS AND DISCUSSIONS:

3.1. Isolation and Identification of Enteric Gram-Negative Bacteria:

All 10 egg shell samples were analysed to detect the presence of enteric gram-negative bacteria using Hektoen Enteric (HE) agar. Initial identification was based on colony morphology and Gram staining. Out of the ten samples, nine showed the presence of red to orange colonies, characteristic of lactose-fermenting bacteria. These colonies were tentatively identified as *Escherichia coli* due to their ability to ferment lactose, which leads to acid production and a corresponding color change on HE agar. One sample exhibited greenish-blue colonies with black centers, a morphology typically associated with hydrogen sulfide (H_2S) production by non-lactose fermenters such as *Salmonella* spp. All isolates, regardless of colony type, were subjected to Gram staining and were found to be Gram-negative rods—consistent with the characteristics of the Enterobacteriaceae family.

Sample	Colony Morphology	Presumptive Identification	Gram Stain Result
1. Barasat	Red/orange colonies	*E. coli*	Gram-negative rods
2. Nalikul 1	Green-blue with black center	*Salmonella* spp.	Gram-negative rods
3. Nalikul 2	Red/orange colonies	*E. coli*	Gram-negative rods
4. Bardhaman 1	Red/orange colonies	*E. coli*	Gram-negative rods
5. Bardhaman 2	Red/orange colonies	*E. coli*	Gram-negative rods
6. Bardhaman 3	Red/orange colonies	*E. coli*	Gram-negative rods
7. Barrackpore 1	Red/orange colonies	*E. coli*	Gram-negative rods
8. Barrackpore 2	Red/orange colonies	*E. coli*	Gram-negative rods
9. Barrackpore 3	Red/orange colonies	*E. coli*	Gram-negative rods
10. Barrackpore 4	Red/orange colonies	*E. coli*	Gram-negative rods

Table 1. Preliminary Identification and Gram Stain Characterization of Isolated Gram-Negative Bacteria

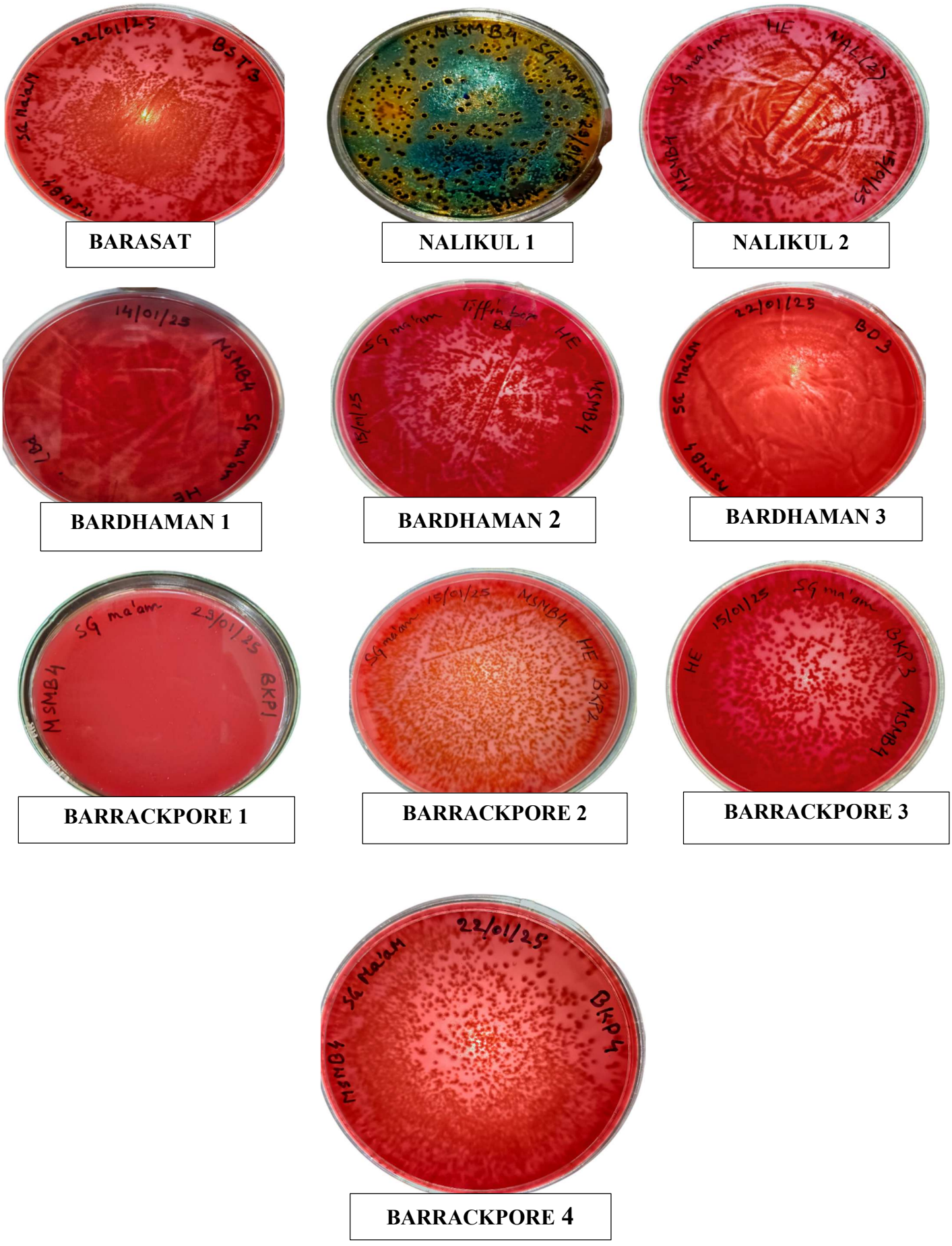


Figure 2: Spread Plates in Hektoen Enteric Agar media

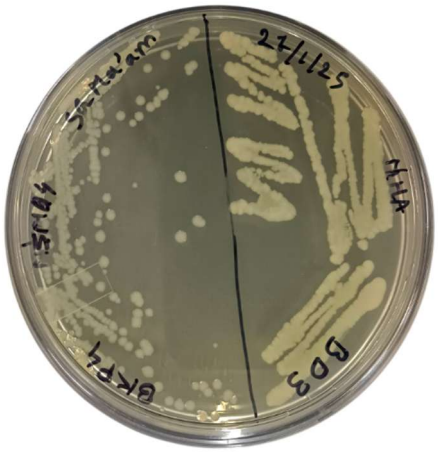
BARASAT

NALIKUL 1 and

BARRACKPORE 1

NALIKUL 2 and BARDHAMAN 2

BARRACKPORE 3 and BARRACKPORE 2



BARRACKPORE 4 and

BARDHAMAN 3

BARDHAMAN 1

Figure 3: Streaking in Muller Hinton Agar media

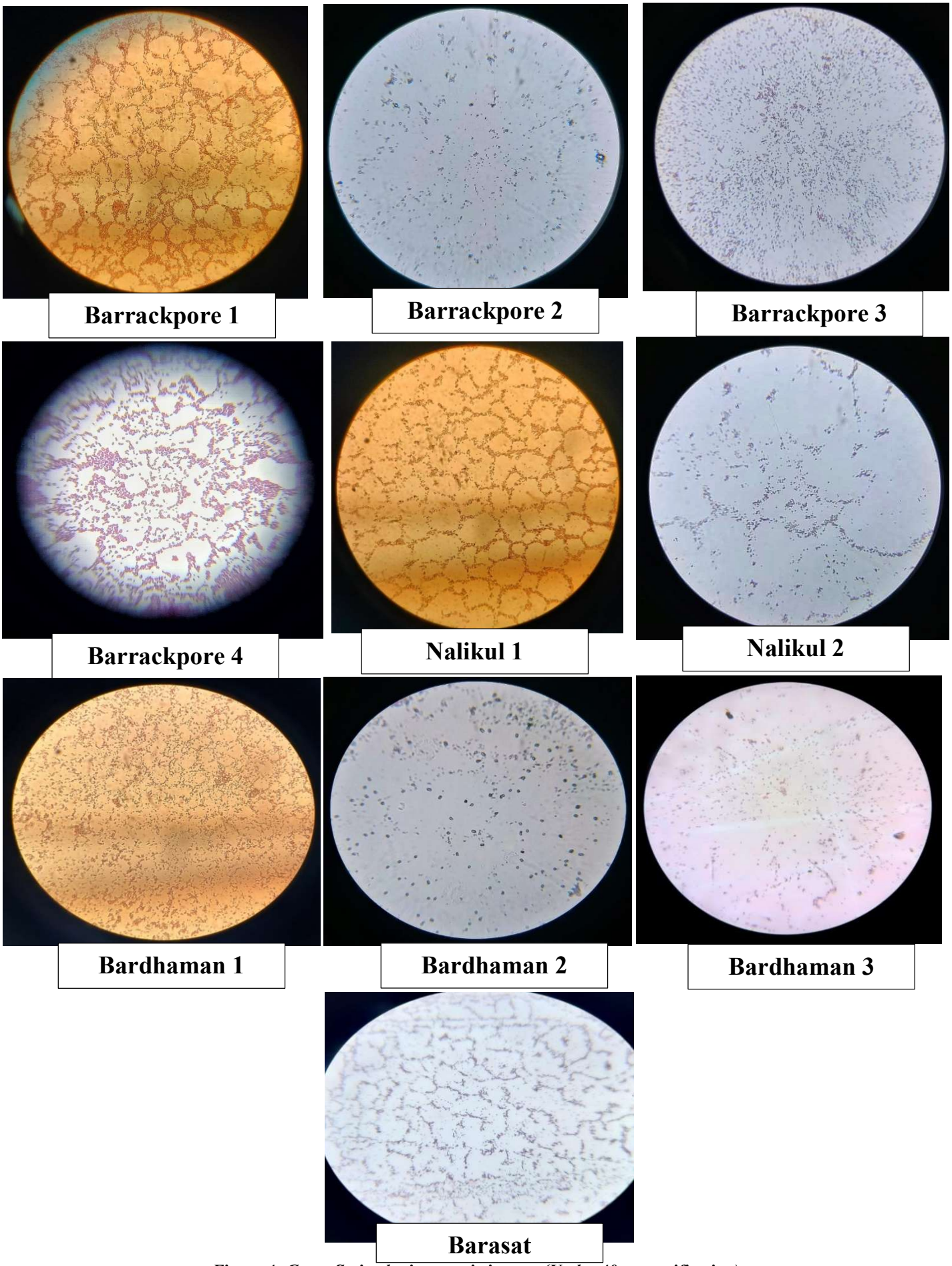


Figure 4: Gram-Stained microscopic images (Under 40x magnification)

3.2. Confirmatory Biochemical Tests:

3.2.1 Triple Sugar Iron (TSI) Test

The Triple Sugar Iron agar test was employed to differentiate *Escherichia coli* and *Salmonella* spp. based on their ability to ferment glucose, lactose, and sucrose, along with gas and hydrogen sulfide (H_2S) production. In most samples, a yellow coloration throughout the slant and butt of the medium, along with gas formation and the absence of black precipitate, indicated acid production from sugar fermentation typical of *E. coli*. In contrast, sample "Nalikul 1" exhibited an alkaline (red) slant, acidic (yellow) butt, gas formation, and a black precipitate at the butt, which is characteristic of *Salmonella* spp. due to H_2S production.

Sample	Media Color Change (Slant/Butt)	Gas Production	Black Precipitate	Presumptive Bacterial Species
1. Barasat	Yellow / Yellow (acidic)	Present	Absent	*Escherichia coli*
2. Nalikul 1	Red / Yellow (alkaline/acidic)	Present	Present	*Salmonella* spp.
3. Nalikul 2	Yellow / Yellow (acidic)	Present	Absent	*Escherichia coli*
4. Bardhaman 1	Yellow / Yellow (acidic)	Present	Absent	*Escherichia coli*
5. Bardhaman 2	Yellow / Yellow (acidic)	Present	Absent	*Escherichia coli*
6. Bardhaman 3	Yellow / Yellow (acidic)	Present	Absent	*Escherichia coli*
7. Barrackpore 1	Yellow / Yellow (acidic)	Present	Absent	*Escherichia coli*
8. Barrackpore 2	Yellow / Yellow (acidic)	Present	Absent	*Escherichia coli*
9. Barrackpore 3	Yellow / Yellow (acidic)	Present	Absent	*Escherichia coli*
10. Barrackpore 4	Yellow / Yellow (acidic)	Present	Absent	*Escherichia coli*

Table 2. Triple Sugar Iron (TSI) Test Results for Confirmation of Bacterial Species

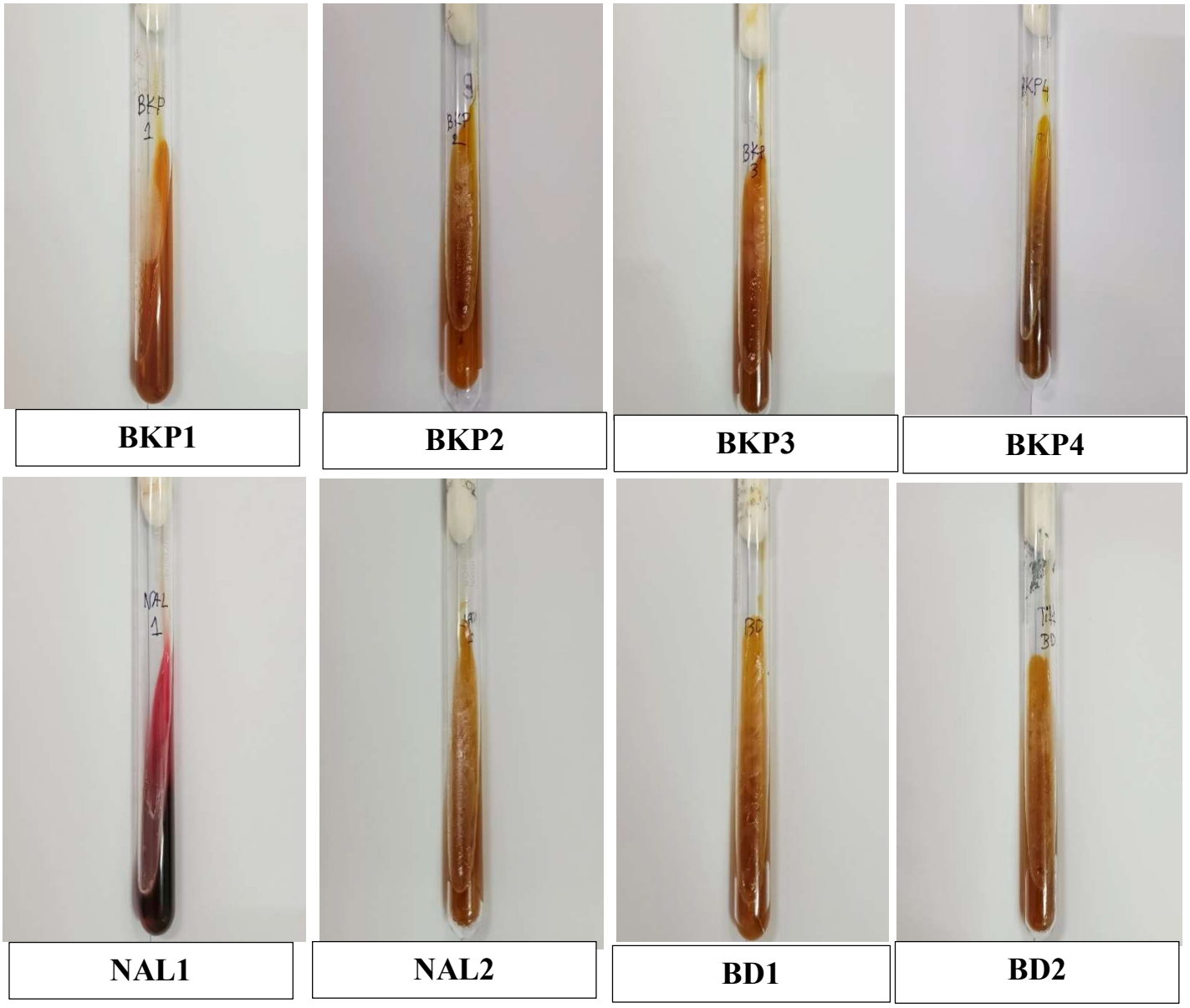


Figure 5: TSI test result

3.2.2. UREASE TEST-

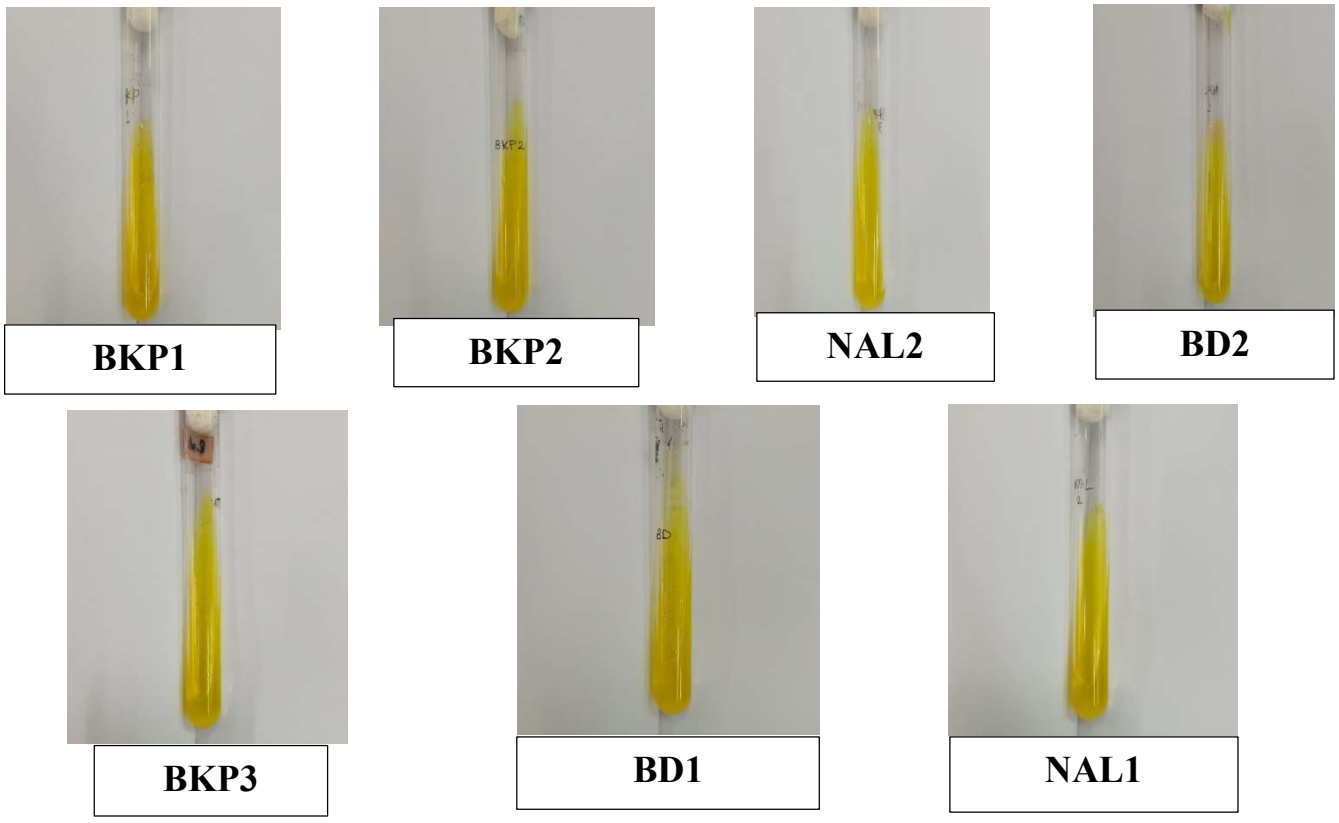
The urease test was conducted to determine the ability of the bacterial isolates to hydrolyze urea using the enzyme urease. None of the tested isolates showed a positive reaction in the urease medium.

- No color change was observed in the medium; it remained light orange to yellow, indicating that the pH remained acidic to neutral (pH < 7).
- No ammonia production was detected, confirming that urea hydrolysis did not occur.
- These results suggest that none of the isolates possess active urease enzyme activity, or if present, it was too weak or slow to be detected under the test conditions.

Table 3: Urease test result

Sample No.	Color Change	Result	Interpretation
1. Barrackpore 1	Yellow	Negative	Urease not produced
2. Barrackpore 2	Yellow	Negative	Urease not produced
3. Nalikul 2	Yellow	Negative	Urease not produced
4. Bardhaman 2	Yellow	Negative	Urease not produced
5. Barrackpore 3	Yellow	Negative	Urease not produced
6. Bardhaman 1	Yellow	Negative	Urease not produced
7. Nalikul 1	Yellow	Negative	Urease not produced

Figure 6: Urease test results



3.2.3. IMVIC TEST-

Table 4: IMVIC test results

Sample No.	Indole	Methyl Red	Voges-Proskauer	Citrate	IMViC Pattern	Identification
1. Bkp 1	+	+	–	–	++--	*E. coli*
2. Bkp 2	+	+	–	–	++--	*E. coli*
3. Bkp 3	+	+	–	–	++--	*E. coli*
4. Bkp 4	+	+	–	–	++--	*E. coli*
5. Bst	+	+	–	–	++--	*E. coli*
6. Bd 1	+	+	–	–	++--	*E. coli*
7. Bd 2	+	+	–	–	++--	*E. coli*
8. Bd3	+	+	–	–	++--	*E. coli*
9. Nal 2	+	+	–	–	++--	*E. coli*
10. Nal 1	–	+	–	+	-+-+	*Salmonella* spp.

The IMViC test series (Indole, Methyl Red, Voges-Proskauer, and Citrate) was employed to further differentiate and confirm the identity of the enteric bacterial isolates.

- **Nine out of ten isolates** displayed the IMViC pattern ++--, indicating positive reactions for indole and methyl red, and negative reactions for Voges-Proskauer (VP) and citrate utilization. This biochemical profile is characteristic of *Escherichia coli*, confirming its presence in these samples.
- **One isolate (Sample 10)** exhibited the IMViC pattern -+-+, showing negative indole, positive methyl red, negative VP, and positive citrate results. This combination is consistent with Salmonella spp., supporting its identification in this sample.

Figure 7: Indole test results

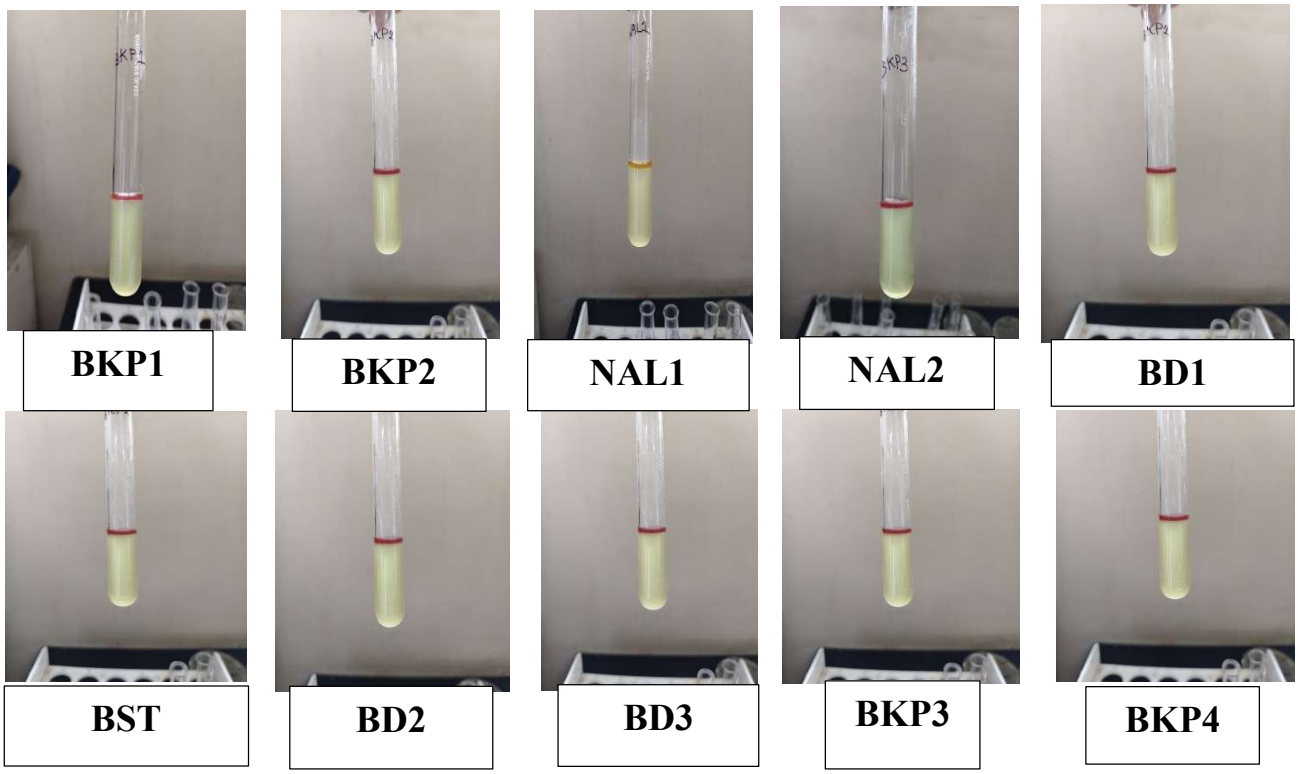


Figure 8: Methyl Red test results

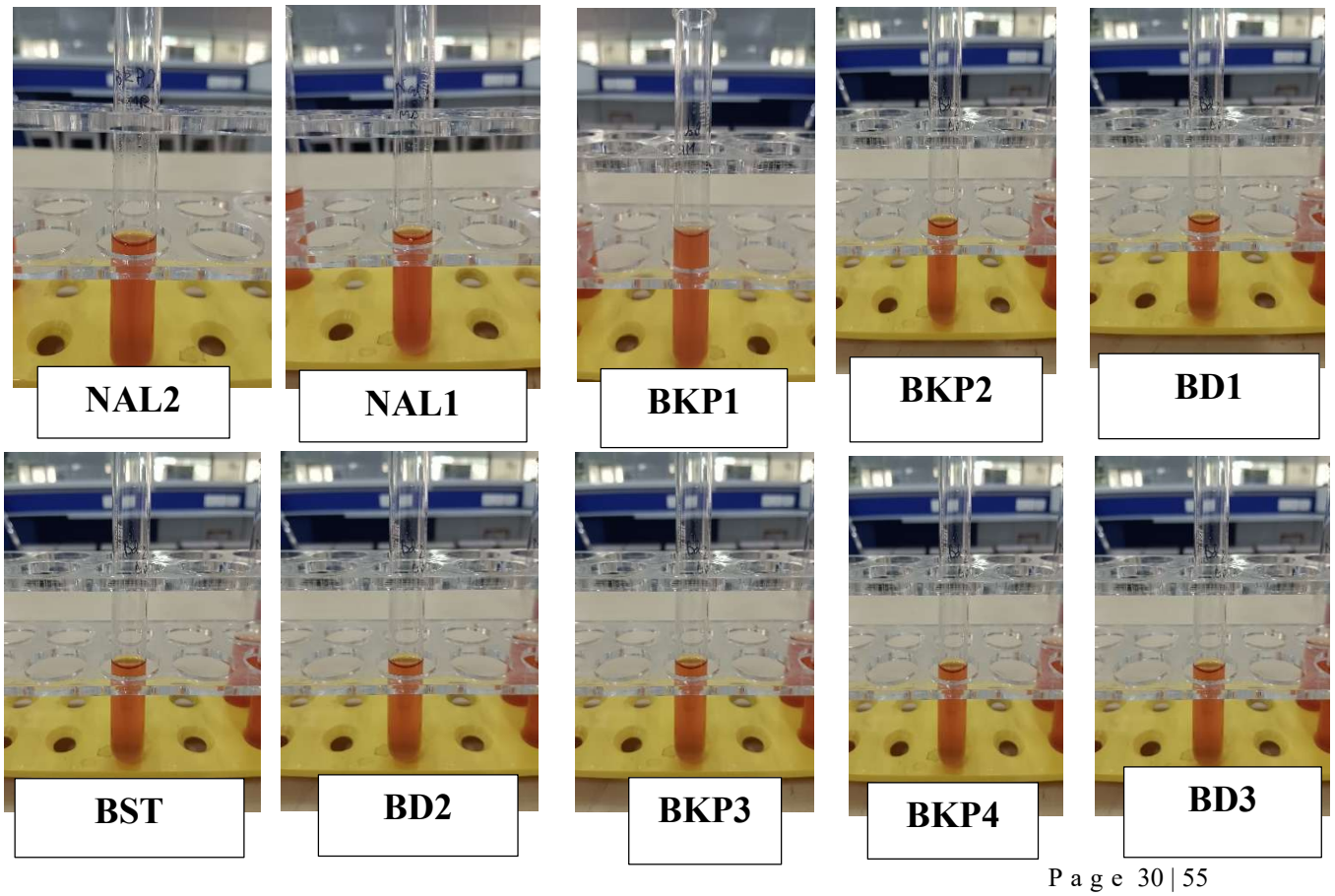


Figure 9: Voges-Proskauer test results

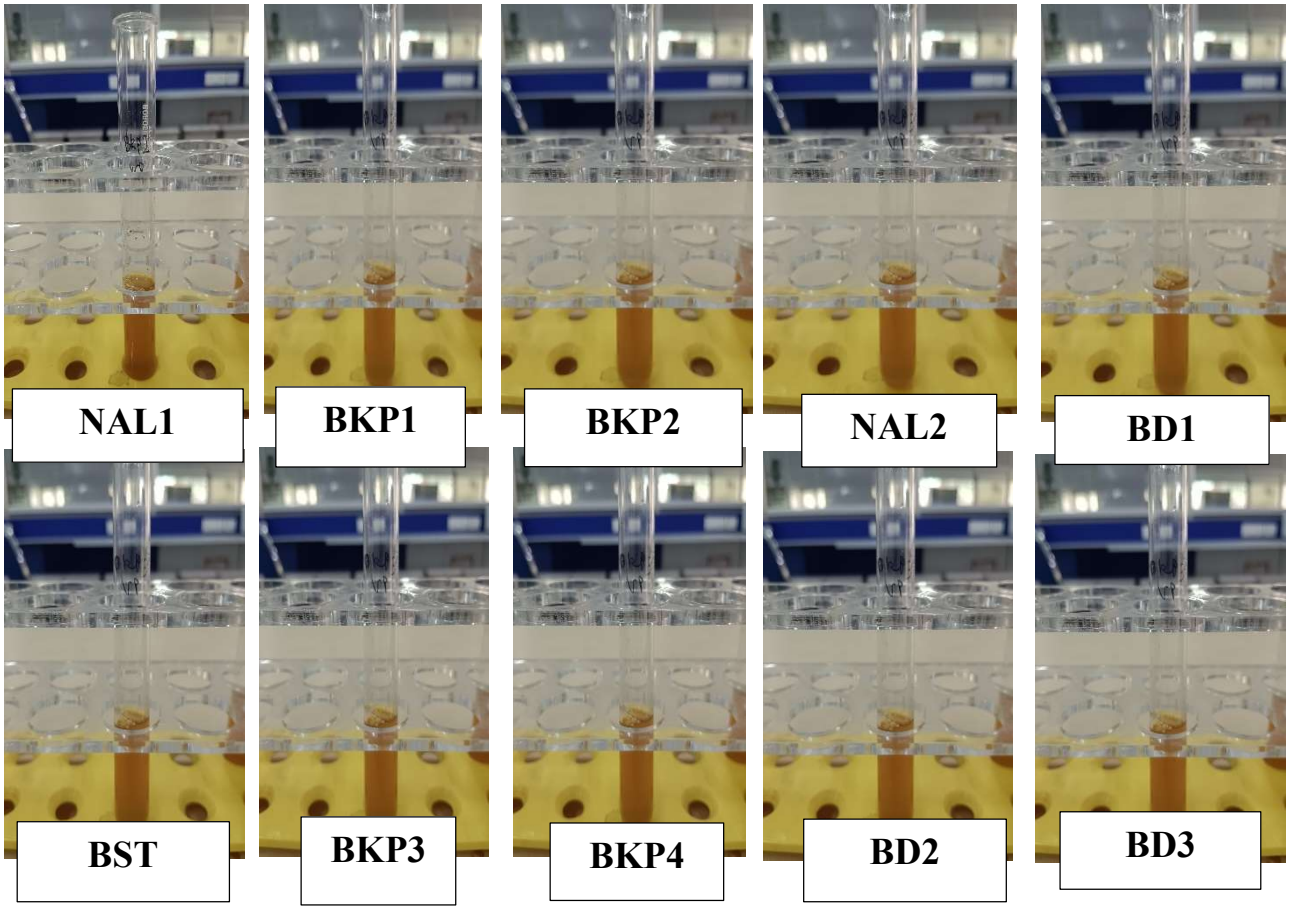
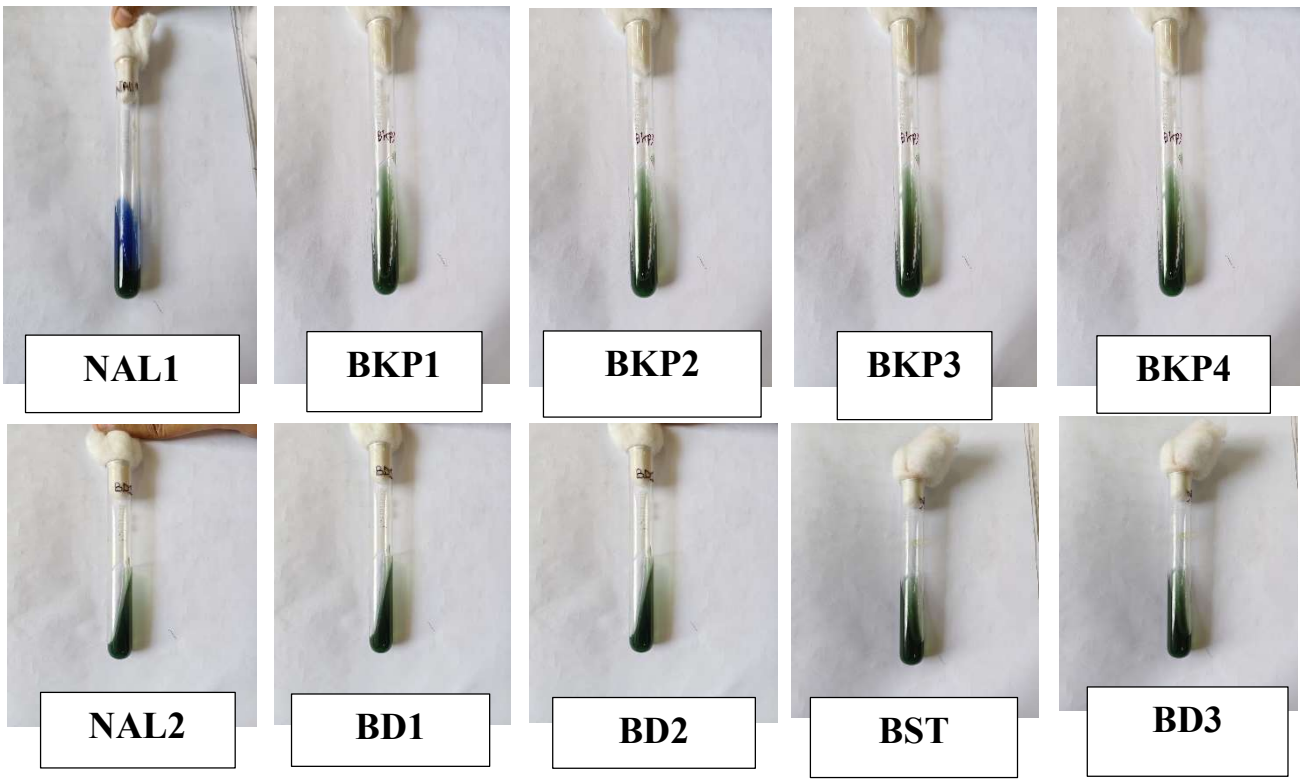


Figure 10: Citrate test results



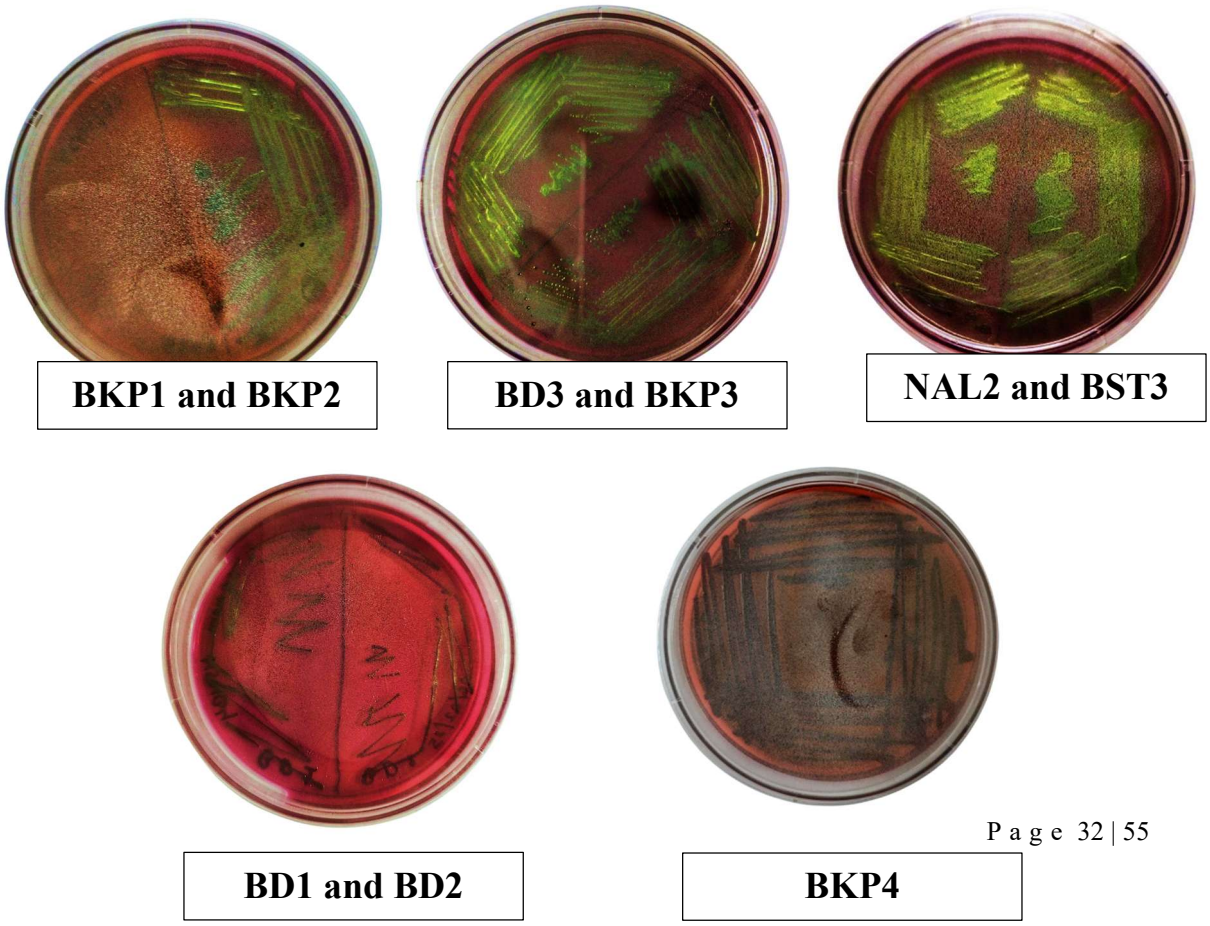
3.2.4. EMB AGAR MEDIA TESTS-

All isolates (BKP1, BKP2, BKP3, BKP4, BD1, BD2, BD3, NAL2, BST3) were streaked on EMB agar plates. The following observations were made:

Table 5: EMB test results

Sample Pair	Colony Appearance	Interpretation
BKP1 & BKP2	**Metallic green sheen**	**Strong lactose fermentation – typical of *E. coli***
BD3 & BKP3	**Green colonies**	**Strong lactose fermenters – *E. coli***
NAL2 & BST3	**Dark greenish metallic colonies**	**Strong lactose fermenters – *E. coli***
BD1 & BD2	**Slight sheen/light greenish tint**	**Slight variation but still consistent with *E. coli***
BKP4	**Lighter or dull colonies**	**Possibly growth variation, still *E. coli***

Figure 11: EMB AGAR MEDIA PLATES-



3.3. ANTIBIOTIC SUSCEPTIBILITY TEST:

BKP1

BST3

NAL1

NAL2

BD

BD2

Figure 12: Antibiotic susceptibility test plates

Table 6: Antibiotic Resistance (+ = Resistant, - = Sensitive)

Location	Kanamycin	Penicillin G	Ampicillin	Chloramphenicol	Streptomycin	Vancomycin	Nalidixic Acid	Gentamycin
BD1	-	-	-	+	+	-	-	+
BD2	-	-	-	+	+	-	-	+
NAL1	-	-	-	+	+	-	-	+
NAL2	-	-	-	-	-	-	-	+
BKP1	-	-	-	+	-	-	-	+
BST3	-	-	-	+	+	-	-	+

Table 7: Diameter of Inhibition Zone (in mm)

Location	Kanamycin	Penicillin G	Ampicillin	Chloramphenicol	Streptomycin	Vancomycin	Nalidixic Acid	Gentamycin
BD1	-	-	-	20 mm	15 mm	-	-	22 mm
BD2	-	-	-	12 mm	13 mm	-	-	26 mm
NAL1	-	-	-	18 mm	14 mm	-	-	18 mm
NAL2	-	-	-	-	-	-	-	10 mm
BKP1	-	-	-	12 mm	-	-	-	22 mm
BST3	-	-	-	10 mm	18 mm	-	-	24 mm

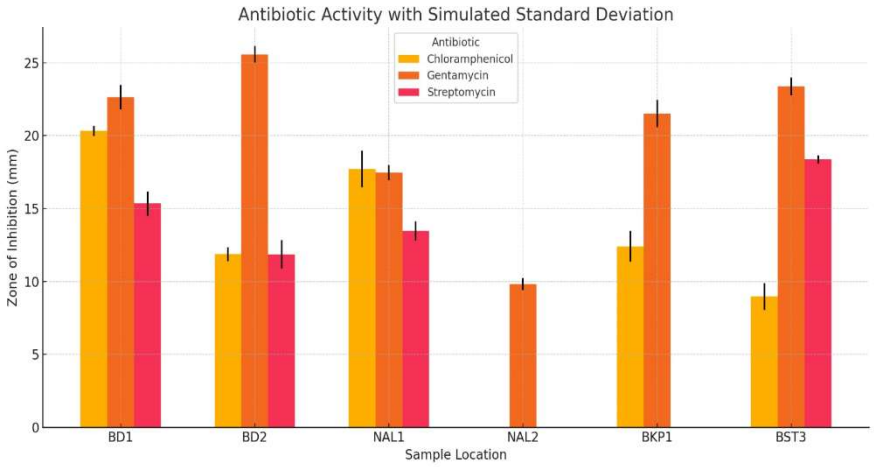


Figure 13: Graphical representation showing the zone of inhibition for different antibiotics across all sample locations. It visually represents how effective each antibiotic was against the isolates from each location.

The present study evaluated the antibiotic susceptibility patterns of enteric bacterial isolates (*Escherichia coli* and *Salmonella* spp.) obtained from environmental samples, including raw egg extracts. Among the antibiotics tested, **Gentamycin** demonstrated the highest and most consistent antibacterial activity across all isolates, with inhibition zones ranging from **10 mm (NAL2)** to **26 mm (BD2)**. This suggests that Gentamycin remains a potent therapeutic agent against both clinical and environmental strains of enteric pathogens. In contrast, **Chloramphenicol** and **Streptomycin** showed **variable efficacy**. While isolates like **BD1**, **NAL1**, and **BST3** displayed moderate sensitivity (10–20 mm zones), **NAL2** exhibited **complete resistance**, indicating a multidrug-resistant (MDR) phenotype. This resistance was further reinforced by the absence of inhibition zones for Penicillin G, Ampicillin, Kanamycin, Vancomycin, and Nalidixic Acid across all isolates. The complete lack of sensitivity in NAL2 to all antibiotics except Gentamycin (and that too at a reduced zone of 10 mm) is particularly concerning, as it reflects a robust resistance mechanism, possibly mediated by **mobile genetic elements** such as plasmids or transposons. Such resistance profiles may be attributed to **environmental antibiotic pressure**, especially in areas like poultry farms, wastewater discharge sites, or egg processing facilities, where antibiotic residues are commonly present. These contaminated environments can foster the **selection and propagation of resistant strains**, contributing to the emergence of AMR in non-clinical settings. The findings also highlight the **limited effectiveness of commonly used antibiotics** (e.g., Penicillin G, Ampicillin, Streptomycin) in environmental isolates, which poses a significant public health threat. The potential of these resistant strains to act as **reservoirs of resistance genes** raises concerns about the **horizontal transfer** of resistance determinants to other microbial populations, including human pathogens. Overall, the study underscores the **urgent need for continuous surveillance and prudent antibiotic use**, not only in healthcare but also in agriculture and environmental management, to combat the growing challenge of antimicrobial resistance.

3.4. ANTIMICROBIAL ACTIVITY OF PLANT (ROSEMARY) EXTRACT:

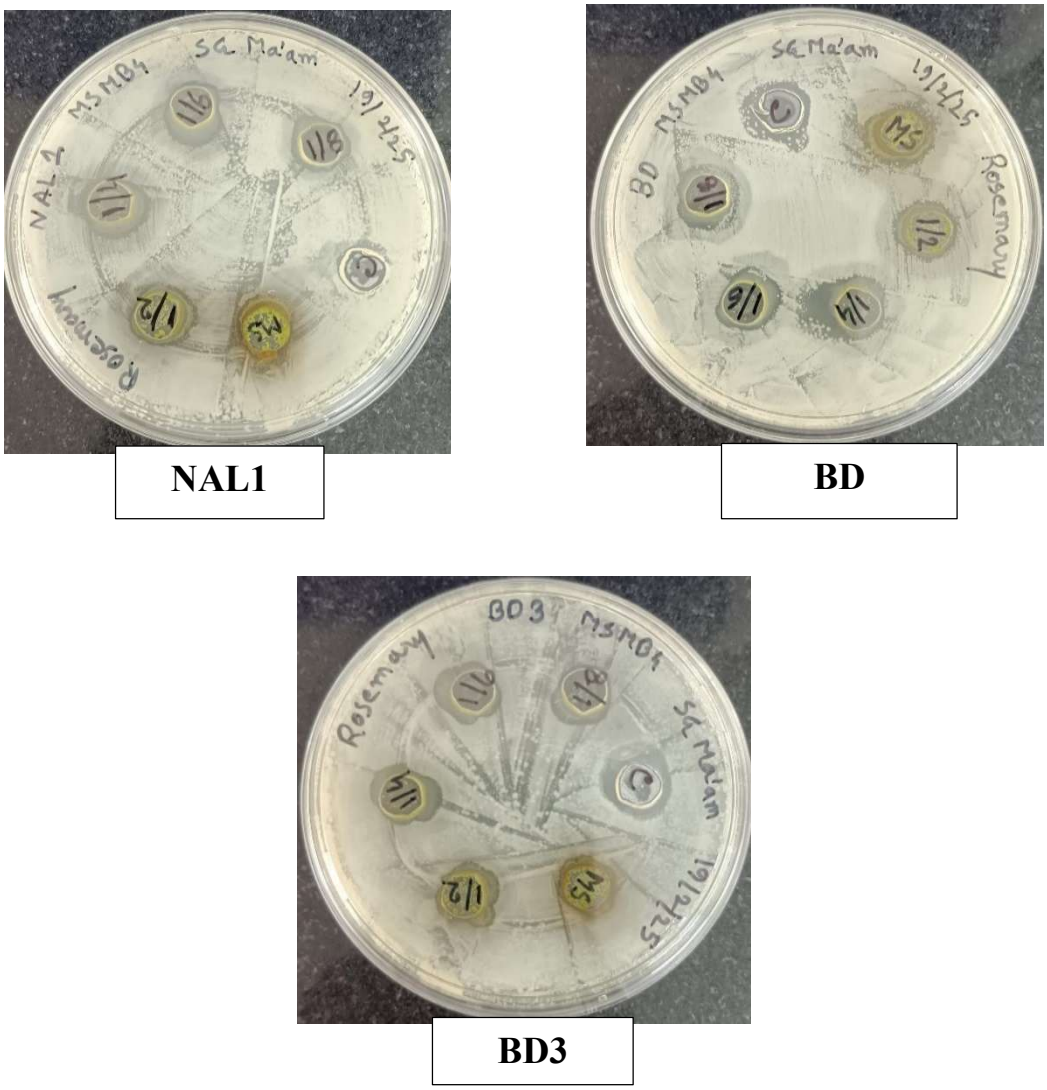


Figure 14: Well diffusion plates

Dilution	BD (*E. coli*)	BD3 (*E. coli*)	NAL1 (*Salmonella*)
Control (C)	18 mm	16 mm	12 mm
Mother Stock	16 mm	12 mm	14 mm
1/2	14 mm	14 mm	14 mm
1/4	24 mm	14 mm	14 mm
1/6	14 mm	14 mm	16 mm
1/8	14 mm	16 mm	14 mm

Table 8: Diameter of Inhibition zone (mm)

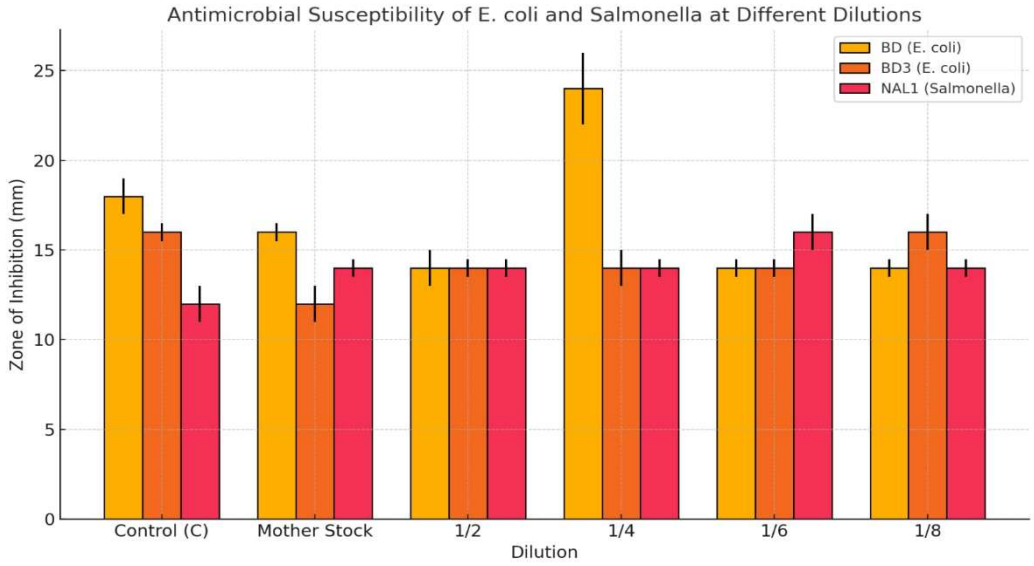


Figure 15: Graphical representation of the antibacterial activity rosemary leaf extract at various dilutions against E. coli (BD, BD3) and Salmonella (NAL1), as measured by the zone of inhibition (in mm).

The rosemary extract demonstrated variable antimicrobial activity depending on the dilution and bacterial strain. Interestingly, *E. coli* BD sample responded most strongly to the 1/4 dilution, indicating a possible synergistic concentration range. In contrast, BD3 sample of E. coli and NAL1 sample of *Salmonella* exhibited moderate but consistent susceptibility. This suggests rosemary extract could be more effective against certain *E. coli* strains at specific concentrations.

3.5. PHYTOCHEMICAL SCREENING OF ROSEMARY LEAF EXTRACT:

3.5.1. QUALITATIVE ANALYSIS:

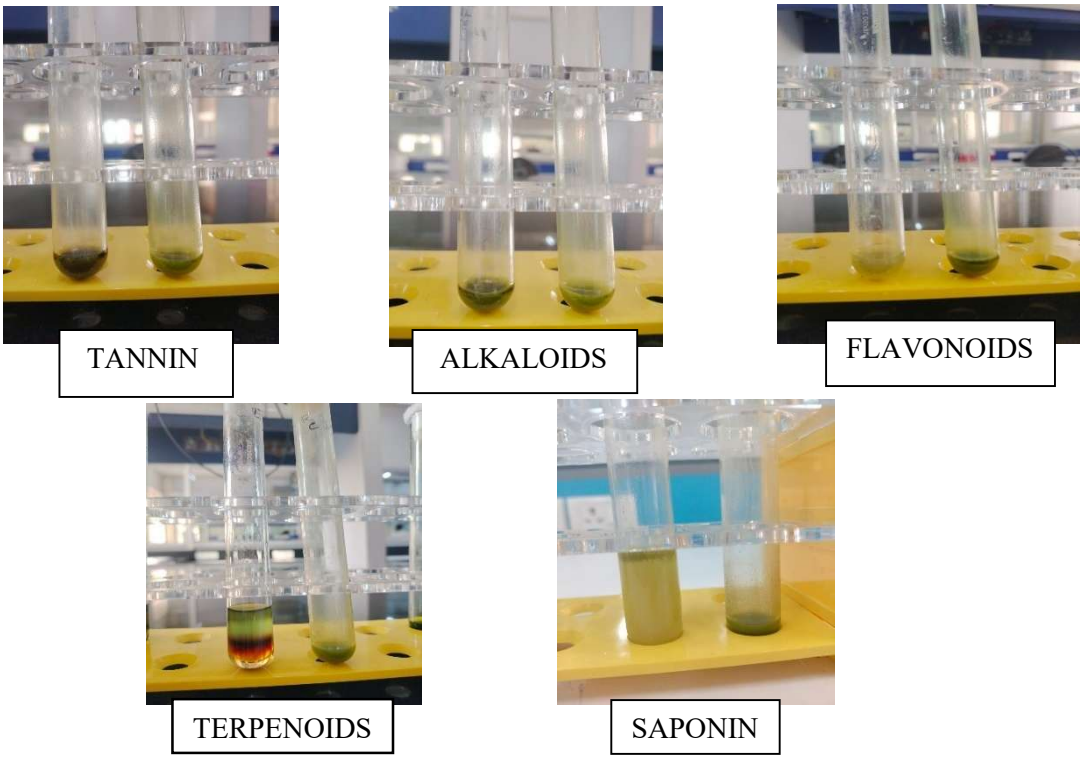


Figure 16: Tannin, Alkaloid, Flavonoids, Terpenoids and Saponin tests results

Figure 15: Presence of Tannin, Alkaloids, Flavonoids, Terpenoids and Saponin in the rosemary leaf extract

Phytochemical	Observation	Inference
Tannin	Formation of dark precipitate at bottom	Tannins present
Alkaloids	Appearance of brown precipitate	Alkaloids present
Flavonoids	White precipitate at the bottom	Flavonoids present
Terpenoids	Reddish-brown interface observed	Terpenoids present
Saponin	Persistent frothing/precipitate formation	Saponins present

Table 9: Qualitative Analysis of Biochemical Constituents in Rosemary Leaf Extract

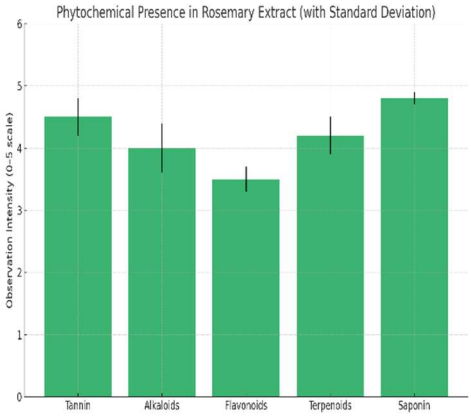


Figure 17: Graphical representation the qualitative presence of phytochemicals in rosemary leaf extract.

The phytochemical investigation of *Rosmarinus officinalis* extract revealed the presence of several significant secondary metabolites, each known for contributing to various therapeutic effects. The detection of **tannins**, indicated by the formation of a dark precipitate, suggests their possible involvement in protein binding and microbial inhibition, which may explain part of the extract's antimicrobial activity. **Alkaloids**, confirmed by a brown precipitate, are pharmacologically active compounds commonly linked to antibacterial, antifungal, and pain-relieving properties. Their presence in rosemary enhances the extract's potential for medicinal application, especially in managing infections and inflammation. The presence of **flavonoids**, observed as a white precipitate, aligns with the well-established antioxidant nature of these compounds. Flavonoids help in protecting cells against oxidative stress and may also support antimicrobial action by disrupting microbial enzymes or cell walls. A distinct reddish-brown interface marked the presence of **terpenoids**, which not only contribute to rosemary's characteristic aroma but also possess a wide range of biological activities including antimicrobial, anticancer, and anti-inflammatory effects. Finally, the appearance of **persistent frothing** in the saponin test indicates their presence. Saponins are known to influence cell membrane permeability, which can enhance the delivery and effectiveness of other phytochemicals. They also have immune-modulating properties and can contribute to the antimicrobial activity observed in the extract. Together, these results underscore the richness of rosemary in bioactive compounds and validate its traditional use in herbal remedies. These compounds likely work synergistically to exert antimicrobial, antioxidant, and therapeutic effects. Further studies focusing on isolation, purification, and mechanism of action of individual constituents could support the development of natural antimicrobial agents or supplements based on rosemary.

3.5.2. QUANTITATIVE ESTIMATIONS:

Figure 18: TPC (Total Polyphenol Content) and DPPH (2,2-diphenyl-1-picrylhydrazyl) tests result

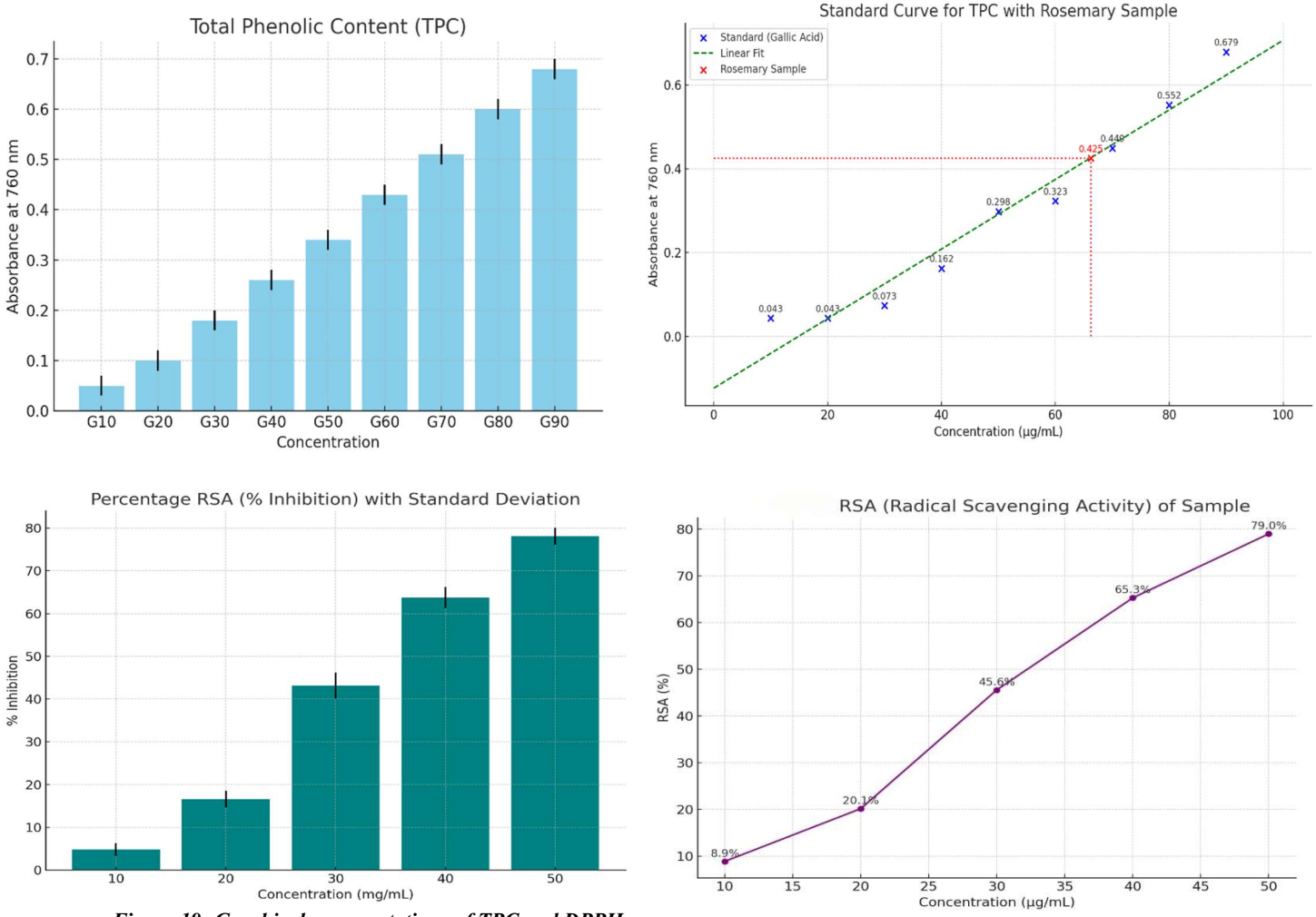


Figure 19: Graphical representations of TPC and DPPH (radical scavenging assay)

The present study aimed to evaluate the total phenolic content (TPC) and antioxidant potential of rosemary leaf extract using the Folin-Ciocalteu method and the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The results obtained from the TPC analysis demonstrated a concentration-dependent increase in absorbance at 760 nm. The absorbance values ranged progressively from lower to higher concentrations (G10 to G90), indicating an increasing presence of phenolic compounds in the extract. The standard curve plotted using gallic acid exhibited a strong linear relationship between absorbance and concentration, which enabled accurate quantification of phenolic content in the rosemary sample. Phenolic compounds are known for their significant antioxidant activity due to their ability to donate hydrogen atoms or electrons and neutralize free radicals. The data from the DPPH radical scavenging assay supported this notion. The percentage inhibition of DPPH radicals increased with increasing concentrations of the extract, showing a peak activity of approximately 74.8% at 50 µg/mL. The gradual rise in % inhibition suggests the dose-dependent scavenging potential of rosemary, further validating its efficacy as a natural antioxidant source. The results of this study confirm that rosemary leaf extract is a rich source of phenolic compounds and possesses potent antioxidant activity. This highlights its potential applicability in pharmaceutical and food industries as a natural antioxidant agent.

3.6. GREEN SYNTHESIS OF SILVER NANOPARTICLES USING ROSEMARY:

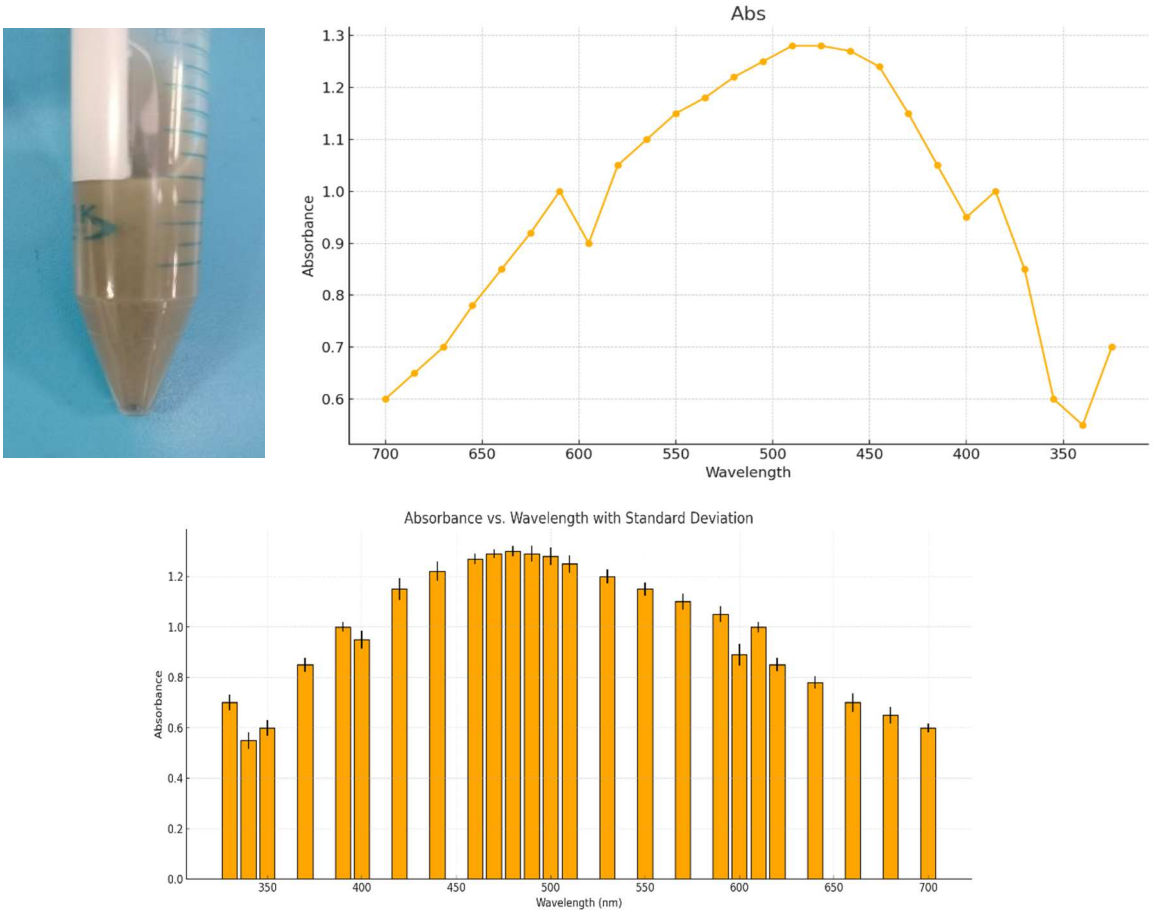


Figure 20: Rosemary silver nanoparticles formation and Graphical representations

The absorbance spectrum obtained confirms the successful formation of silver nanoparticles, as indicated by the distinct SPR peak around 480 nm. This peak corresponds to the collective oscillation of conduction electrons on the nanoparticle surface, a known optical signature for silver nanoparticles of spherical or near-spherical morphology. The presence of low standard deviations around the peak region indicates consistent and stable nanoparticle synthesis. Slight variations at the tails of the wavelength range (above 650 nm and below 350 nm) suggest minor fluctuations, possibly due to background interference or instrumental sensitivity at extreme wavelengths. Rosemary extract is rich in phytochemicals such as flavonoids, terpenoids, and phenolic compounds, which likely functioned as both **reducing agents**, converting Ag^{+} ions to metallic Ag^{0}, and **stabilizing agents**, preventing aggregation of the nanoparticles. The well-defined SPR peak and minimal error margins in the core absorption range reflect efficient nanoparticle stabilization by these bioactive compounds. These findings are consistent with previous reports on green synthesis of silver nanoparticles, highlighting the eco-friendly potential of plant extracts in nanotechnology. The consistency in the spectral profile across multiple replicates further supports the reliability of rosemary extract as a biogenic medium for silver nanoparticle production.

3.7. ANTIMICROBIAL ACTIVITY OF ROSEMARY LEAF EXTRACT AND NANOPARTICLE:

Figure 21: Well diffusion plates with plant extract and nanoparticle

Dilution	BD (*E. coli*)	BD3 (*E. coli*)	NAL1 (*Salmonella spp.*)
Control (Ethanol)	12 mm	20 mm	14 mm
NP 1/2	34 mm	16 mm	32 mm
NP 1/8	18 mm	18 mm	24 mm
R 1/8	16 mm	12 mm	20 mm

Table 10: Diameters of Inhibition zone (mm)

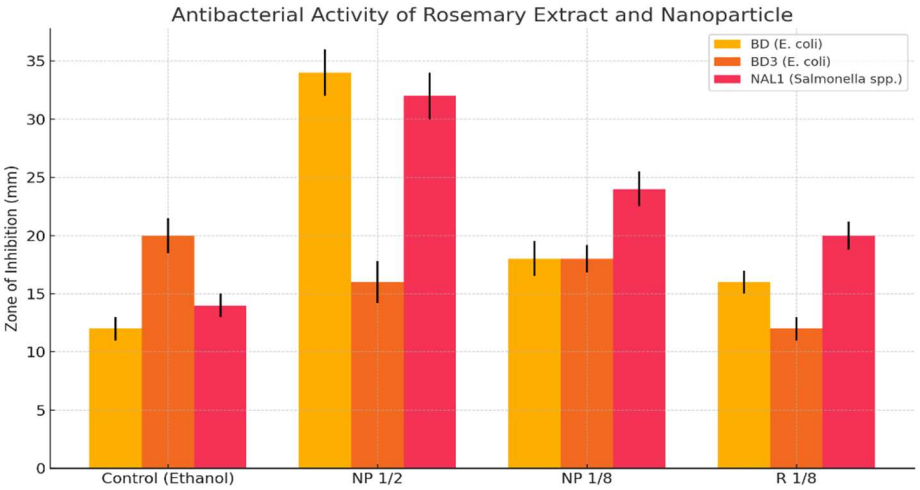


Figure 22: Graphical representation of the antibacterial activity rosemary leaf extract and nanoparticle at various dilutions against E. coli (BD, BD3) and Salmonella (NAL1), as measured by the zone of inhibition (in mm).

The results indicate that both rosemary extract and its nanoparticle formulations exhibit notable antimicrobial activity against E. coli strains (BD and BD3) and Salmonella spp. (NAL1). Among all treatments, the nanoparticle at half concentration (NP 1/2) demonstrated the strongest antibacterial effect, particularly against E. coli BD and Salmonella spp., showing zones of inhibition of 34 mm and 32 mm respectively. This suggests enhanced efficacy of the nanoparticle form, possibly due to increased surface area and improved penetration into bacterial cells. Interestingly, the NP 1/8 dilution still showed significant activity, especially against Salmonella spp. (24 mm), implying that even at lower concentrations, the nanoparticles retain considerable antibacterial potential. The regular rosemary extract at the same dilution (R 1/8) had reduced effectiveness, especially against E. coli BD3, where the inhibition zone was only 12 mm. The control (ethanol) exhibited minimal inhibition, confirming that the observed antibacterial effects are primarily due to the rosemary components rather than the solvent. The difference in response between bacterial strains also highlights potential differences in their cell wall structures or susceptibility to the active compounds. Overall, these findings support the potential of rosemary-based nanoparticles as effective antibacterial agents, especially for food safety or medical applications where broad-spectrum activity is desired.

3.8. INHIBITION OF BIOFILM FORMATION BY ROSEMARY EXTRACT AND NANOPARTICLES:

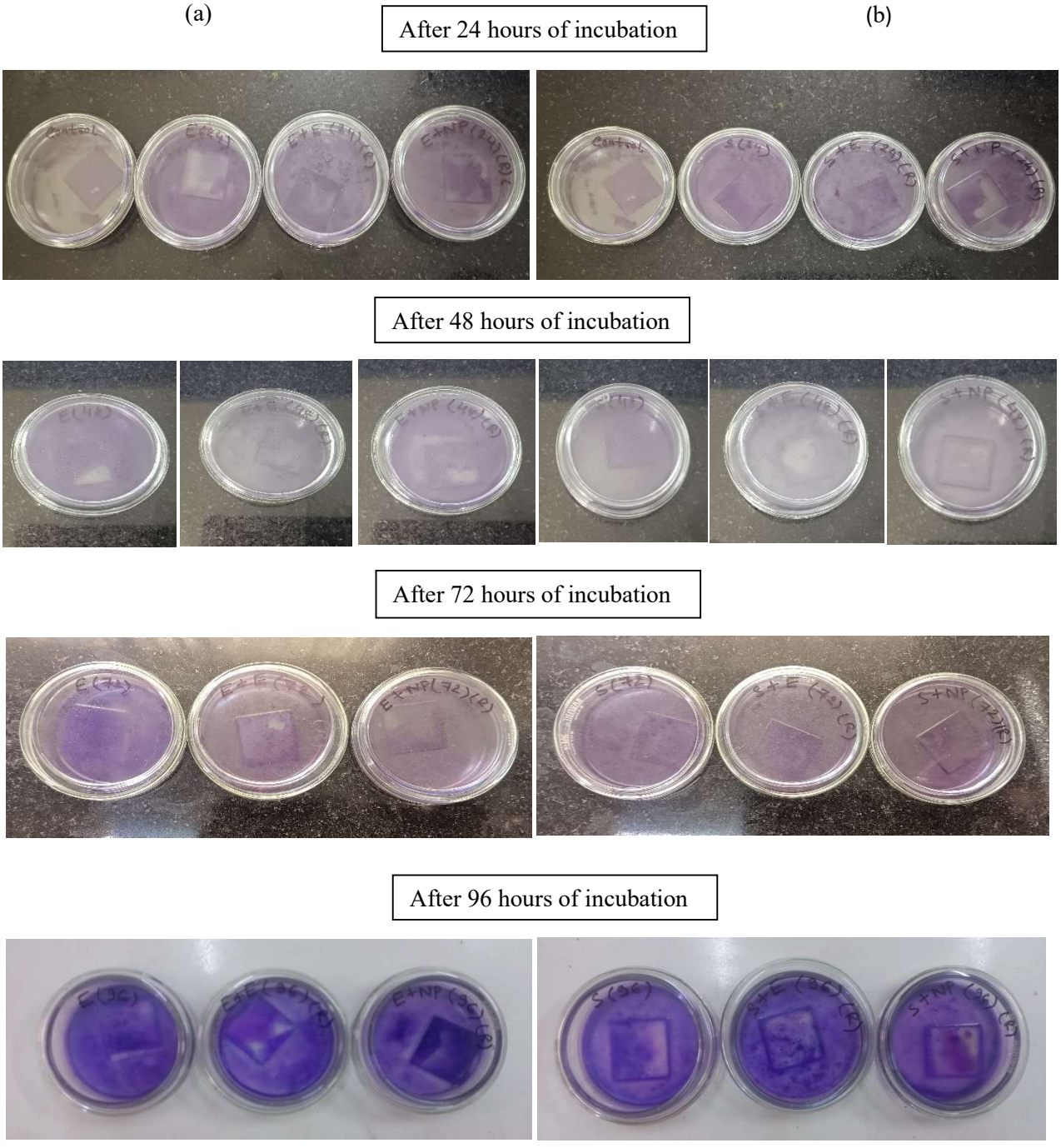


Figure 23: Biofilm formation observation plates (a)E. coli, (b) Salmonella spp.

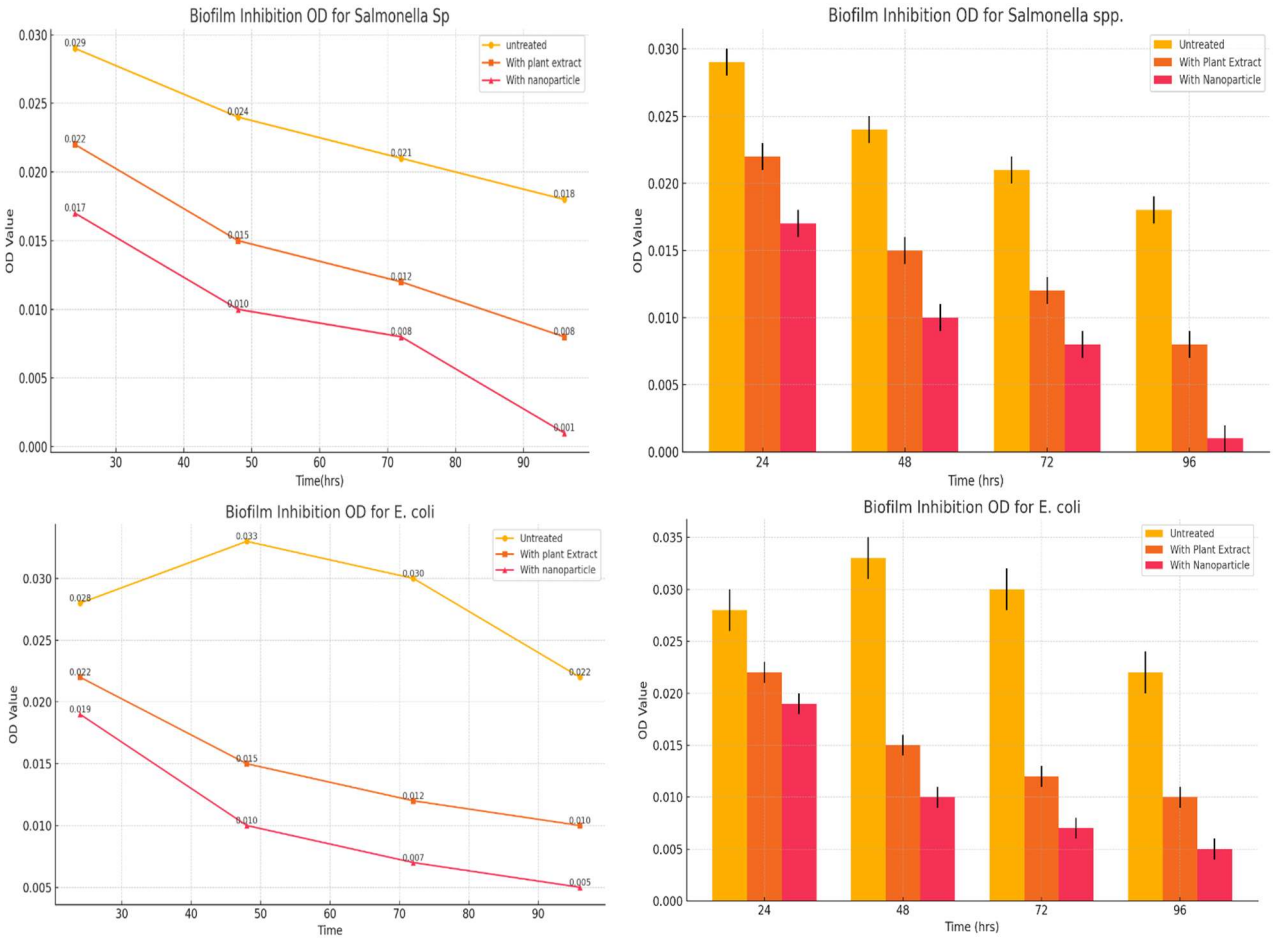


Figure 24: Graphical representations of biofilm formation - Salmonella spp. and E. coli

The results demonstrate that rosemary nanoparticles have a significant impact on the inhibition of biofilm formation in both *Salmonella spp.* and *E. coli*. Across all time points, the treatment with nanoparticles consistently resulted in the lowest optical density (OD) values, indicating the highest degree of biofilm suppression. In contrast, untreated samples showed the highest OD values, confirming robust biofilm development in the absence of treatment. In the case of *Salmonella spp.*, the OD values for the nanoparticle-treated samples decreased progressively from 0.017 at 24 hours to just 0.001 at 96 hours. This steep decline suggests that the nanoparticles not only inhibited initial biofilm formation but also disrupted its persistence over time. The plant extract treatment also reduced biofilm development compared to the control, though to a lesser extent, with a final OD of 0.008 at 96 hours. A similar trend was observed for *E. coli*, where nanoparticle treatment led to a steady reduction in OD values from 0.019 to 0.005. Notably, untreated *E. coli* exhibited a temporary increase in OD at 48 hours, peaking at 0.033 before declining. This peak suggests a typical biofilm maturation phase, which was significantly curtailed in the treated samples. The superior effectiveness of the nanoparticles over the crude plant extract may be attributed to their smaller size and greater surface area, allowing better interaction with bacterial cells and enhanced penetration into biofilms. These findings align with previous research that supports the use of nanoparticles in overcoming bacterial defense mechanisms, including biofilm formation. Overall, the study underscores the potential of rosemary-based nanoparticles as promising agents for managing biofilm-associated infections. Further work could explore the mechanisms of action and assess their effectiveness in real-world settings or in combination with conventional antimicrobials.

3.9. DISRUPTION OF PREFORMED BIOFILMS BY ROSEMARY EXTRACT AND NANOPARTICLES:

Figure 25: Preformed biofilm observation plates (a)E. coli, (b) Salmonella spp.

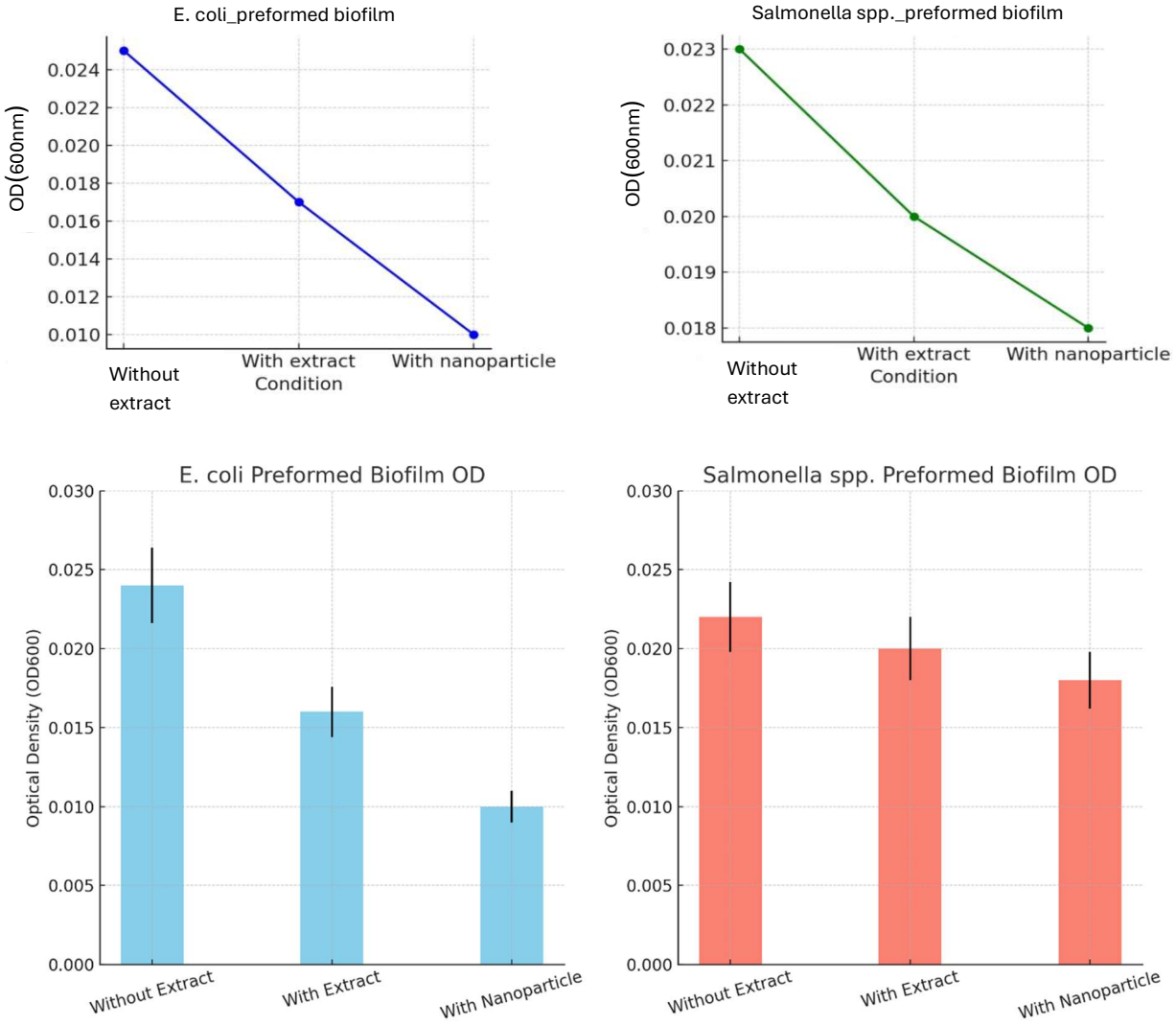


Figure 26: Graphical representations of Preformed biofilm (E. coli and Salmonella spp.)

The comparative analysis of preformed biofilm optical densities (OD_{600}) reveals a clear reduction in biofilm mass upon treatment with both rosemary leaf extract and its synthesized silver nanoparticles. In both *E. coli* and *Salmonella spp.*, the highest OD_{600} values were observed in the untreated control groups, indicating robust biofilm formation under standard conditions. Treatment with rosemary extract alone resulted in a noticeable decrease in OD_{600}, suggesting that the phytochemical constituents of the extract possess antibiofilm properties. This effect was more pronounced in *E. coli* compared to *Salmonella spp.*, possibly due to species-specific differences in cell surface structures and biofilm matrix composition. The most significant reduction in OD_{600} was recorded in groups treated with silver nanoparticles synthesized using rosemary extract. This highlights the synergistic effect of metal nanoparticles and phytochemicals in disrupting established biofilms. The enhanced antibiofilm activity may be attributed to the nanoparticles' ability to penetrate biofilm layers and generate reactive oxygen species, leading to structural damage and microbial cell death.

4. CONCLUSION:

This study underscores the significant public health threat posed by the consumption of raw eggs, particularly due to contamination with enteric pathogens such as *Escherichia coli* and *Salmonella* spp. The isolation of antibiotic-resistant strains from these samples further highlights the escalating issue of antimicrobial resistance (AMR) and the diminishing effectiveness of conventional antibiotic therapies. These findings point to an urgent need for the development of alternative antimicrobial strategies.

Among potential solutions, plant-derived compounds offer promising natural interventions. In particular, rosemary (*Rosmarinus officinalis*) extract has demonstrated notable antimicrobial properties. When utilized in combination with nanotechnology—specifically, for the green synthesis of silver nanoparticles (AgNPs)—its antibacterial and antibiofilm activities are significantly enhanced. In this study, rosemary-mediated AgNPs exhibited strong inhibitory effects against both *E. coli* and *Salmonella* isolates. This synergistic approach not only improves the therapeutic potential of plant extracts but also represents a sustainable and eco-friendly method for addressing foodborne infections.

The results advocate for the broader integration of plant-based, green-synthesized nanoparticles into public health initiatives and food safety management systems. By reducing dependence on synthetic antibiotics, such strategies may play a crucial role in slowing the spread of AMR. Furthermore, the application of phytochemical-nanoparticle combinations offers exciting prospects for future research, potentially expanding into therapeutic innovations and environmental antimicrobial solutions. Overall, the study highlights the necessity of continued exploration into natural product-based nanotechnologies, promoting safer, more effective methods to combat resistant microbial pathogens and enhance global health outcomes.

5. APPENDIX:

Appendix I: Sample Collection Details

Location	Number of Samples Collected
Barasat	1
Nalikul	2
Bardhaman	3
Barrackpore	4

Appendix II: Media and Reagents Utilized

- **Hektoen Enteric Agar (HEA)** – Selective isolation of enteric pathogens.
- **Mueller Hinton Agar (MHA)** – Antibiotic susceptibility testing.
- **Eosin Methylene Blue (EMB) Agar** – Differentiation of lactose fermenters.
- **Triple Sugar Iron (TSI) Agar** – Biochemical identification of enteric bacteria.
- **Urea Agar Slants** – Detection of urease production.
- **Simmons Citrate Agar** – Assessment of citrate utilization.
- **Tryptone Broth** – Indole production test.
- **MR-VP Broth** – Methyl Red and Voges-Proskauer tests.
- **Folin-Ciocalteu Reagent** – Estimation of total phenolic content.
- **DPPH (2,2-diphenyl-1-picrylhydrazyl)** – Evaluation of antioxidant activity.
- **Silver Nitrate Solution (1 mM)** – Green synthesis of silver nanoparticles.

Appendix III: Analytical Techniques

- **Gram Staining** – Morphological and Gram character determination.
- **Biochemical Testing** – TSI, Urease, IMViC, and EMB agar tests for bacterial confirmation.
- **Antibiotic Susceptibility Testing** – Kirby-Bauer disc diffusion method.

- **Phytochemical Screening** – Detection of tannins, alkaloids, flavonoids, terpenoids, and saponins.
- **Total Phenolic Content (TPC) Assay** – Quantification of phenolics via UV-Vis spectrophotometry.
- **DPPH Radical Scavenging Assay** – Assessment of antioxidant potential.
- **Nanoparticle Synthesis and Characterization** – Visual observation and UV-Vis spectroscopy (Surface Plasmon Resonance peak analysis).
- **Biofilm Formation and Inhibition Assays** – Using crystal violet staining and OD600 measurement.

Appendix IV: Instruments and Equipment

- Laminar airflow cabinet
- Hot air oven
- Autoclave
- UV-Vis spectrophotometer
- Colorimeter
- pH meter
- Refrigerated centrifuge
- Shaking incubator
- Incubator (37°C)
- Light microscope with oil immersion objective (40x)
- Electronic blender
- Analytical balance
- Sterile Petri plates, pipettes, conical flasks, and sterile filtration setup

Appendix V: Preparation of Extracts and Nanoparticles

- **Rosemary Leaf Extract Preparation**: 2 g powdered leaves extracted with 20 mL absolute ethanol; filtered and stored at 4°C.
- **Green Synthesis of Silver Nanoparticles**: Aqueous rosemary extract mixed with 1 mM $AgNO_3$ solution in 1:1 ratio; color change monitored as evidence of nanoparticle formation.

Appendix VI: Summary of Bacterial Isolates

Sample Source	Presumptive Organism Identified
Barasat	*Escherichia coli*
Nalikul 1	*Salmonella* spp.
Nalikul 2	*Escherichia coli*
Bardhaman 1-3	*Escherichia coli*
Barrackpore 1-4	*Escherichia coli*

Appendix VII: Observations on Antibacterial and Antibiofilm Activities

- **Rosemary leaf extract** showed moderate antibacterial activity against *E. coli* and *Salmonella* spp.
- **Green-synthesized silver nanoparticles** exhibited significantly enhanced antibacterial and antibiofilm activity.
- **Zones of inhibition** were largest at 1/2 nanoparticle dilution compared to crude extract treatments.
- **Biofilm inhibition** was notably higher with nanoparticle treatment across all time points compared to untreated controls

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